Exploring the potential health benefits from *Hermetia illucens* and *Chrysomya chloropyga* larvae meal in poultry diets

by

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Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Summary

The unaffordability and scarcity of good quality protein sources are especially severe for small scale farmers in rural areas of Africa. For this reason, the monogastric animal feed industry is in urgent need of new and sustainable protein sources. Insects have been proposed as a sustainable, high-quality protein source. A major focus has been placed on *Hermetia illucens* (BSF) (Diptera: Stratiomyidae) larvae due to their ability to break down organic matter from waste streams and convert it into high-quality protein. However, research on carrion species such as *Chrysomya chloropyga* (CC) (Diptera: Calliphoridae) larvae, which are excellent in converting animal offal, is scarce. Even though numerous trials have proven that larvae meal can be used as a protein source in monogastric animal diets, the following question remains: Is this novel protein source only good to use as a protein source in animal diets to sustain production, or does it hold other benefits? Since published data regarding the immunomodulatory and antimicrobial properties of larvae meal in poultry diets are limited, this study focused on the effects of BSF and CC larvae meals on some of these properties when used in the diets of broiler chickens and broiler quails. The larvae meal sources used in this study provided essential amino acid profiles close to the requirements of broilers. Larvae meal from both Diptera species was accepted by broilers when used in a diet preference trial. Three animal trials were conducted for this study to determine the immunomodulatory and antimicrobial properties of larvae meal. In the first trial, BSF and CC larvae meals were used in the diets of 108 broiler female chickens over a 35-day growth period. Larvae meal was added to the diets at inclusion levels of 10% and 15%. Results were compared with broilers receiving a maize-soya-fishmeal based control diet (CON) or a control diet supplemented with an antimicrobial growth promoter, Zinc Bacitracin (ZincBac). The weekly measured production parameters for all treatment groups were similar to that of the control group, except for broilers in the 15%BSF group which had a poorer feed conversion ratio (FCR) at 35 days of age. Broilers were injected with sheep red blood cells and phytohaemagglutinin-P (PHA-P) to determine the effect of dietary treatments on the humoral immune response and cell-mediated immune response in the form *T-cell lymphoproliferation*. Increased antibody titers against sheep red blood cells and a greater swelling response to PHA-P were detected in broilers receiving BSF and CC larvae meal sources. Treatments had no negative effects on haematological parameters, lymphoid organ weights, liver colour, or gastrointestinal pH. Based on these results, it was concluded that the BSF and CC larvae meals were non-toxic and had no negative effect on the physiological status of the broiler chickens. For the second trial, BSF larvae were reared on two different substrates: 100% commercial layer chicken mash (BSF-M), or 50% commercial layer chicken mash + 50% fish offal (BSF-F). Resulting larvae were used as feed for quail. This trial aimed to determine the effects of larvae meal on specific immune parameters and selected bacterial counts in the quail ceca. Fish offal was chosen to form part of the larvae's substrate to increase the content of long-chain omega-3 (n-3) fatty acids in the larvae meal. Sixty quails were injected with porcine red blood cells, and PHA-P. Quails in the BSF-F group exhibited lower slaughter weight compared to quails in the CON and BSF-M group. Quails in the BSF-M group had a significantly higher secondary humoral immune response compared to the CON group. Dietary inclusion of larvae meal significantly increased lymphoproliferative response, with the BSF-F group

exhibiting the greatest response. Dietary treatments had no effect on *in vivo* serum bactericidal activity against *E. coli*. Most serum protein fractions were not influenced by treatment, with the exception of α2-globulin being higher in the BSF-M and BSF-F groups, whereas γ-globulin concentrations were lower in the serum of the BSF-F group. It was concluded that larvae meal has immunomodulatory properties in broiler quails, but the substrate used to rear the larvae can influence the results. In a third trial, a challenge experiment with *Salmonella enterica* serovar Enteritidis A9 was conducted. A total of 476 broiler chickens were orally challenged with *Salmonella* Enteritidis. Broilers received either a control diet (CON+SAL), control diet supplemented with oxytetracycline antibiotic growth promoter (ANTIBIO+SAL), a diet containing 10% CC larvae meal (CC+SAL) or 10% BSF larvae meal (BSF+SAL). One group of broilers received the control diet but was not infected with *Salmonella* and served as the negative control group (CON-NEG). One bird per cage (replicate) was slaughtered on day 11, 14, 21, 24 and 28 for ceca and blood collection. Feed conversion ratio of chickens in the CC+SAL, BSF+SAL and ANTIBIO+SAL treatment groups were significantly improved compared to the CON+SAL treatment group. Since FCR were similar between broilers receiving larvae meal and broilers receiving the antimicrobial growth promoter, it is possible that 10% larvae meal can replace the need for antibiotic growth promoters in broiler diets since it delivered similar results in challenged animals. Oxytetracycline significantly reduced *Salmonella* colonisation one- and four-days post-infection, but ceca *Salmonella* levels slowly increased again over time in this treatment group. The opposite was noticed for the CC+SAL group, with CFU per ceca counts slowly decreasing until being significantly lower than the CON+SAL group on day 28. *Salmonella* counts were similar in the BSF+SAL and CON+SAL groups on all the slaughter days. Both larvae meal sources significantly enhanced serum bactericidal activity against *Salmonella* when compared to the CON+SAL group. Lymphoproliferative response to the PHA-P test was significantly higher in the CC+SAL, BSF+SAL and ANTIBIO+SAL treatment groups; whereas only the BSF+SAL group had enhanced lysozyme concentrations in their blood shortly after infection occurred. Lastly, treatments had no effect on lymphoid organ weight, haematological parameters, or serum interferon-gamma (IFN- γ) levels of broilers. To summarise the results from the three trials; BSF and CC larvae meals showed promising immunostimulating properties in broiler chickens and quails - dietary larvae meal showed signs of an increased humoral immune response, T lymphocyte function and serum lysozyme activity in both animal species. Even though BSF larvae meal did not change cecal microbial composition against selected bacterial counts in quails, when challenged with *Salmonella* Enteritidis, dietary CC larvae meal exhibited antimicrobial properties by decreasing *Salmonella* colonisation in the ceca as well as increasing serum bactericidal activity against the challenged organism. Even though no difference in FCR was observed in this study when healthy broilers or quails received larvae meal, there was an indication that larvae meal could improve FCR in infected animals, since BSF and CC larvae meal improved FCR in *Salmonella* infected broilers. To conclude, all the immune parameters studied in these trials were either improved or similar for poultry receiving dietary larvae meal, but the larvae species, as well as the substrate used to rear the larvae on, may affect the response.

Opsomming

Die onbekostigbaarheid en skaarsheid van goeie gehalte proteïenbronne is 'n probleem vir kleinskaalse boere in die landelike gebiede van Afrika. Die monogastriese veevoerbedryf het dus 'n dringende behoefte vir nuwe en volhoubare proteïenbronne. Insekte is voorgestel as alternatiewe proteïenbronne. Die fokus is tans op *Hermetia illucens* (BSF) larwes, vanweë hul vermoë om organiese afval om te skakel na proteïene van 'n hoë gehalte. Navorsing op aasvlieë, soos bv. *Chrysomya chloropyga* (CC) larwes, wat uitstekend is in die omskakeling van diere-afval, is egter skaars. Alhoewel talle navorsing studies bewys het dat larwemeel as 'n proteïenbron kan dien in monogastriese diëte, bly daar steeds baie vrae onbeantwoord. Tans is dit onbekend of larwemeel slegs goed is as proteïenbron in voer, en of dit dalk ook ander voordele inhou. Gepubliseerde data oor die immunomodulatoriese en antimikrobiese eienskappe van larwemeel in pluimveediëte is beperk. Hierdie studie het dus gefokus om die immunomodulatoriese en antimikrobiese eienskappe van BSF en CC larwemeel wat in braaikuiken- en kwarteldiëte gevoeg was, te bepaal. Die larwemeel bronne wat gebruik was in hierdie studie het die nodige aminosuurprofiele gehad wat voldoen het aan die moderne braaikuiken se behoeftes. Larwemeel van beide Diptera spesies is deur braaikuikens aanvaar tydens 'n voorkeur proef. Drie diereproewe was uitgevoer om die immunomodulatoriese en antimikrobiese eienskappe van larwemeel te bepaal. In die eerste proef was BSF of CC larwemeel in die diëte van 108 braaikuikens ingesluit tydens 'n 35 dae groeiperiode. Insluitingsvlakke van 10% en 15% larwemeel was tydens die studie getoets. Die resultate was vergelyk met resultate van braaikuikens wat 'n kontrole dieet (CON) ontvang het. Resultate was ook vergelyk met resultate van hoenders wat die kontroledieet, aangevul met 'n antimikrobiese groei promotor, Zink Bacitracin (ZincBac), ontvang het. Weeklikse produksieparameters vir alle behandelings groepe was soortgelyk aan die kontrole groep s'n, behalwe vir braaikuikens in die 15% BSF-groep, wat op 35 dae 'n swakker voeromsettingsverhouding gehad het. Tydens die studie was braaikuikens met skaap rooibloedselle en fitohaemagglutinien-P (PHA-P) ingespuit om die effek van dieetbehandelings op die humorale en sel-gemediëerde immuunresponse te bepaal. Verhoogde teenliggaam titers en 'n groter swelling reaksie was waargeneem in braaikuikens wat BSF en CC larwemeel ontvang het. Behandelings het geen negatiewe effekte op enige van die bloed parameters, orgaangewigte, lewerkleur of gastro-intestinale pH gehad nie. Op grond van hierdie resultate is die gevolgtrekking gemaak dat BSF en CC larwemeel nie giftig is nie, en so ook geen negatiewe uitwerking op die fisiologie van braaikuikens het nie. Vir die tweede proef is BSF-larwes op twee verskillende substrate geproduseer; 100% kommersiële lêhen meel (BSF-M), of 50% kommersiële lêhen meel + 50% visafval (BSF-F). Die doel van hierdie studie was om die effek van larwemeel toediening op spesifieke immuunparameters en bakteriese tellings in die sekum van kwartels te bepaal. Visafval is gekies om die inhoud van langketting-omega-3 (n-3) vetsure in die larwemeel te verhoog. Sestig kwartels is met vark rooibloedselle asook PHA-P ingespuit. Kwartels in die BSF-F groep het laer slaggewigte gehad in vergelyking met kwartels in die CON en BSF-M groepe. Kwartels in die BSF-M groep het 'n hoër sekondêre humorale immuunrespons gehad in vergelyking met kwartels in die CON groep. Die insluiting van larwemeel in die voer het so ook die limfoproliferatiewe respons verhoog en kwartels in die BSF-F groep het die grootste reaksie getoon. Dieet behandelings het geen effek gehad op die antibakteriese werking van die serum teen *E. coli* nie. Die meerderheid van die proteïenfraksies

in die serum van kwartels was nie beïnvloed deur die verskillende behandelings nie, met die uitsondering van α2-globulien wat hoër was in die BSF-M en BSF-F groepe, terwyl γ-globulienkonsentrasies laer was in die serum van kwartels in die BSF-F-groep. Dit was bevind dat larwemeel immunomodulatoriese eienskappe besit, en dat die substraat waarop die larwes geproduseer is hierdie eienskappe kan beïnvloed. Tydens 'n derde proef is 'n uitdagings eksperiment met *Salmonella enterica* serovar Enteritidis A9 uitgevoer. Altesaam was 476 braaikuikens mondelings met *Salmonella* Enteritidis geïnfekteer. Braaikuikens het óf 'n kontrole dieet (CON+SAL), óf 'n kontrole dieet aangevul met ’n antibiotiese groeistimulant (ANTIBIO+SAL), óf 'n dieet wat 10% CC larwemeel (CC+SAL), of 10% BSF larwemeel (BSF+SAL) bevat, ontvang. 'n Groep braaikuikens het so ook die kontrole dieet ontvang, maar is nie geïnfekteer met *Salmonella* nie, die groep was genaamd die negatiewe kontrole groep (CON-NEG). Een braaikuiken per hok is op dag 11, 14, 21, 24 en 28 geslag om sodoende hul bloed en sekums te versamel. Die voeromsetverhouding (VOV) van braaikuikens in die CC+SAL, BSF+SAL en ANTIBIO+SAL behandelingsgroepe was betekenisvol beter as die van die CON+SAL groep. Aangesien soortgelyke VOV aangeteken was tussen braaikuikens wat larwemeel en antimikrobiese groei promotors ontvang het, is dit moontlik dat 10% larwemeel die antibiotiese groei promotors in braaikuikendiëte kan vervang. Oksitetrasikliene het *Salmonella*-kolonisasie op dag een- en dag vier na infeksie aansienlik verminder, maar die *Salmonella*-vlakke in die sekum van hoenders het mettertyd weer gestyg. Die teenoorgestelde is opgemerk vir die CC+SAL-groep. Tot en met dag 28 het die *Salmonella* tellings in die sekum stadig afgeneem totdat dit beduidend laer was as die van die CON+SAL groep. *Salmonella* tellings was dieselfde in die BSF+SAL en CON+SAL groepe op alle slagdae. Beide larwemeel bronne het die serum se antibakteriese aktiwiteit teen *Salmonella* verhoog in vergelyking met die CON+SAL groep. Die limfostimulerende respons was ook aansienlik hoër in die CC+SAL, BSF+SA en ANTIBIO+SAL behandelingsgroepe. Braaikuikens wat BSF ontvang het, het hoër serum lisosiem konsentrasies gehad kort na infeksie met *Salmonella*. Die verskillende behandelings het geen effek op orgaan gewigte, bloed parameters of serum interferon-gamma (IFN-γ) vlakke gehad nie. Ter opsomming: BSF en CC larwes het belowende immunostimulerende eienskappe getoon in braaikuikens en kwartels. ’n Verhoogde humorale immuunrespons, T-limfosiet funksie en serum lisosiem aktiwiteit is aangeteken in pluimvee wat larwemeel onvang het. Alhoewel BSF-larwemeel nie die samestelling van mikrobes in die sekum van kwartels verander het nie, het CC-larwemeel wel antimikrobiese eienskappe teen *Salmonella* in die sekum getoon, gepaard met ’n verhoging in serum antimikrobiese aktiwiteit wanneer braaikuikens met *Salmonella* geïnfekteer was. Alhoewel larwemeel geen verskil in die VOV in gesonde braaikuikens of kwartels veroorsaak het nie, het dit wel ‘n beduidende verbetering veroorsaak in hoenders wat met *Salmonella* besmet was. Alle immuunparameters wat bestudeer was tydens die studie het óf verbeter, óf dieselfde gebly, maar die larwespesies, sowel as die substraat wat gebruik word om die larwes op te groei, kan die voordelige eienskappe beïnvloed.

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Notes

The language and style used in this dissertation are in accordance with the requirements of the *South African Journal of Animal Science*. This dissertation represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters is therefore unavoidable.

The following papers have been published from this thesis:

Pasotto, D., van Emmenes, L., Cullere, M., Giaccone, V., Pieterse, E., Hoffman, L.C., Dalle Zotte, A. (2020). Inclusion of Hermetia illucens larvae reared on fish offal to the diet of broiler quails: effect on immunity and cecal microbial populations. Czech Journal of Animal Science, 65(06): https://doi.org/10.17221/60/2020-CJAS

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Chapter 1

Introduction

The demand for animal protein intended for human consumption is continuously increasing, resulting in a higher demand for livestock production. Since the demand for livestock is increasing, the demand for feed is also increasing. Therefore, a steady supply of sustainable protein sources to be used in the animal feed industry is required. The energy component usually forms the largest part of a monogastric diet, whilst protein sources make the second-largest contribution. The quality of the protein source and its usefulness in a diet depends on four main factors: the protein concentration (g/kg) in the source, its amino acid composition, the digestibility of the amino acids, and lastly, the antinutritional factors present within the source (Burton *et al.*, 2014). A large portion of the protein used in monogastric animal diets is plant-based and mostly derived from oilseeds. However, the antinutritional factors within plant-derived protein sources can cause several detrimental effects. Common effects are usually suppressed feed intake and a reduction in growth, but in severe cases, tumour induction can take place (Makkar, 1993; Burton *et al.*, 2014).

As mentioned above, the primary protein sources used in monogastric animal feeds are oilseed derived protein sources such as soya bean meal, sunflower meal, canola meal etc. In 2019, a total of 347Mt of soya was produced globally, whereas the sum of the other oilseeds totalled to 154 Mt (FAO 2020). South Africa has a minimal contribution to the global soya figure and is expected to produce only 1.5 million tonnes of soya in 2019/2020 (USDA 2019). Even though 94% of the protein used in monogastric animal feed is derived from oilseeds, the 6% contribution from animal-by-products and fishmeal plays a vital role in balancing the amino acid profile of the diet.

As mentioned above, the value of a protein source to a specific animal depends on digestibility of the amino acids and how closely the amino acid profile matches the amino acid requirements of the animal. The digestibility of essential amino acids in fishmeal and soya bean meal is similar. Except for cysteine, the true digestibility coefficient for the essential amino acids in fishmeal and soya bean meal ranges between 88-92%. Whereas the digestibility of poorer quality sources such as feather meal and cottonseed meal ranges between 66-85% and 67-87%, respectively (NRC, 1994).

The protein found in cereal grains like maize and wheat, which is most commonly used in monogastric diets, are deficient in lysine and methionine. Soya bean meal and other legume proteins contribute towards the lysine requirement of the animal; however, these sources are limiting in sulphur-containing amino acids (methionine and cysteine). Even though the animal's requirements for methionine and cysteine can be met by increasing the soya bean meal inclusion in the diet, it will be costly. The factor that makes fishmeal so attractive as a protein source is its balanced amino acid composition. The balanced amino acid profile of fishmeal complements plant-based diets and allows for the formulation of a more nutrient-dense diet. Fishmeal is palatable, and a small dietary inclusion level will usually enhance feed intake in young animals and improve nutrient uptake and absorption (Karimi, 2006; Miles & F.A, 2015). Fishmeal also contains high levels of lysine, methionine and cysteine (NRC, 1994; National Research Council, 1998; Miles & F.A, 2015). Therefore, a low inclusion level of

fishmeal in a diet can more effectively balance the amino acid composition of the diet to meet the requirements of the animal.

Fishmeal also contains essential and beneficial fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which in turn helps to maintain a healthy immune system (Miles & Chapman, 2015). Unfortunately, the increase in the market price for fishmeal has resulted in overfishing, which in turn reduced the quantity of fish that can be obtained from the ocean (Wijkstrom, 2009). Plant-derived protein sources used in animal feed is also receiving criticism due to its environmental impact. An increase in industrial farming systems is needed to meet the demand for feed and food, and as a result, deforestation and habitat loss increases. Furthermore, the use of edible plant proteins in animal feed, instead of its use for human consumption (food), has also caused controversy from an ethical perspective (Henchion *et al.*, 2017). Therefore, the need for sustainable, good quality protein sources, that is free of antinutritional factors and does not create feed-food competition is evident.

The difficulties that the poultry industry experiences to maintain low production costs have resulted in the industry revaluating alternative protein sources. As a result, the opportunity to research insects, one of nature's natural protein sources for poultry, was stimulated. Even though research on the utilisation of insects as a protein source was done in the past, the need for such work was never as pressing as now. One of the advantages of using fly (Diptera) larvae meal as an alternative protein source for monogastric animals is the potential of larvae to be used in waste management. Fly larvae can be used to harvest nutrients from wastes (food, manure, abattoir waste, etc.) without risking environmental microbial contamination or greenhouse gas production (van Zanten *et al.*, 2010; Oonincx *et al.*, 2010; Smetana *et al.*, 2015). A large amount of research and trials have been reported on to produce a novel sustainable protein source from fly larvae by means of nutrient recycling from waste. Various insect species have been explored for their use in feed and food, with *Hermetia illucens* (Linnaeus, 1758; Diptera: Stratiomyidae) (BSF) larvae being one of the most popular species studied. Research is scarce on carrion Diptera species, such as *Chrysomya chloropyga* (CC), which is excellent in converting animal offal (abattoir waste).

Infection due to foodborne pathogens represents a considerable burden in both developing and developed countries. Efforts to reduce transmission of these pathogens through food must be implemented. Studies have shown that fly larvae exhibit antibiotic characteristics and several antimicrobial peptides have been extracted from fly larvae for medicinal purposes (Leem *et al.*, 1999; Meylaers *et al.*, 2004; Park *et al.*, 2014). If the antimicrobial peptides in fly larvae are still active in larvae meal, it may decrease the pathogen load in the gastrointestinal tract of animals, decreasing the possibility of carcass contamination during slaughtering. Many countries are moving away from the use of antimicrobials for disease control and growth promotion in animals due to the emergence of bacterial resistance. Various biological and medicinal activities of extracts from house fly larvae (*Musca domestica*), have been reported (Meylaers *et al*., 2004, Hou, Shi *et al*., 2007, Ai, Wang *et al*., 2013, Cao, Xu *et al*., 2009). A few research papers have been published on immunomodulatory properties of BSF larvae meal as well as other insect meals (Henry *et al.*, 2018; Taufek *et al.*, 2018; Xiao *et al.*, 2018;

Yu *et al.*, 2020).These results indicate that the use of insects in animal diets could have additional benefits such as immunostimulation and *in vivo* antimicrobial activity.

Even though numerous trials have proven that larvae meal can be used as a protein source in monogastric animal diets, the following question remains: Is this novel protein source only good to use as a protein in poultry diets to sustain production, or does it hold other benefits not yet determined? Therefore, this study aimed to evaluate the potential benefits of BSF and CC larvae meal when used in poultry diets. The objectives of this study were to determine if the meal from CC larvae and BSF larvae (reared on different substrates) can be successfully used in broiler and quail diets, and to determine the potential immunomodulatory and antimicrobial properties of these insect meals.

To fulfil the objectives of this study, three animal trials were conducted using BSF or CC meal in the diets of broiler chicken and broiler quails.

- The first trial determined the effect of BSF and CC larvae meal on humoral immune response and cellular immune response by challenging broiler chicken with sheep red blood cells and phytohaemagglutinin-P, respectively. Effects on growth parameters, haematological traits and organ indices were determined.
- For the second trial, BSF larvae were reared on two substrates, one containing fish offal and the other substrate containing only chicken feed. The aim was to increase the omega-3 (n-3) fatty acids in BSF larvae to ultimately determine if n-3 induced larvae meal will provoke different immunomodulatory and antimicrobial properties in broiler quails. Humoral and cellular immune response, serum lysozyme activity, serum bactericidal activity, serum biochemical indexes and selected cecal microbial populations were determined.
- In the last trial, broilers were challenged with *Salmonella enterica* serovar Enteritidis A9 to determine the immunomodulatory and *in vivo* antimicrobial properties of BSF and CC larvae meal. The objectives were addressed by challenging the broilers twice with *Salmonella enterica* subsp. *enterica* serovar Enteritidis A9 through oral gavage. Production parameters and mortality rate were recorded. Broilers were slaughtered at different time-periods to determine the effects on cecal *Salmonella* counts, serum bactericidal activity, serum lysozyme concentrations, serum IFN-γ concentrations, haematological parameters and lymphoid organ weights.

If secondary benefits from these two larvae meal sources in poultry diets can be established through these trials, it may promote acceptance and demand for larvae meal by farmers, feed companies and consumers. This will ultimately support and encourage the need for large scale larvae production and lessen the need for fishmeal and soya bean meal.

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Chapter 2

Literature Review

2.1 The use of insect meal in animal feed

Even though food security/scarcity is prevailing in many developing countries, roughly one-third of edible food that is produced globally for human consumption is being discarded in the form of waste. This accounts for approximately 1.3 billion tonnes of wasted food per year (FAO 2011). This waste occurs throughout the supply chain, starting at the initial agricultural production systems and ending at the consumer. For this reason, there is a great demand for recycling food waste into consumable food or feed. As several insect species can be reared on bio-waste streams; rearing insects, and using insects, in their various morphological development stages as a food or feed source, has become a topic of interest over the past few years. Entomophagy, the act of eating insects, has been practised for centuries and takes place predominantly in Asia, Africa and Latin America, and serves as a supplement in the diets of at least two billion people (Bukkens, 1997; Makkar *et al.*, 2014). Out of almost one million described insect species, 2000 of them were documented as being consumed by humans (Barroso *et al.*, 2017).

Insects form part of the diet of several animal species in the wild, but the use of insects in the diets of livestock is still a relatively new concept. Even though research on the use of insects in poultry diets dates back to the ’70s (Teotia & Miller, 1973; Gawaad & Brune, 1979; DeFoliart, 1989) and has been proposed as a sustainable, high-quality protein source (Bosch *et al*., 2014); it is not yet being used on a scale to make an impact on the need for other protein sources. One factor that makes large scale rearing of insects so attractive is their efficiency in converting substrate into body mass. Considering feed conversion ratio; crickets are twice as efficient as poultry, four times more efficient compared to swine, and 12 time more efficient than cattle (Van Huis, 2013). From an environmental perspective, insect farming is more environmentally friendly compared to other high-quality protein farming systems. The production of insect protein produces less greenhouse gas compared to conventional livestock systems, uses considerably less land compared to soya plantations, and is not accompanied with high energy usage plus greenhouse gas emissions like fishmeal production are (van Zanten *et al*., 2010; Oonincx *et al.*, 2010; Smetana *et al.*, 2015).

Different insect species have diverse feeding habits and can be fed on several waste streams such as restaurant waste, manure, cereal by-products, or offal from slaughterhouses (Nguyen *et al.*, 2015; Parry, 2017). Even though there are approximately one million species of insects, the nutrient composition of only a tiny fraction of the species has been determined (Sánchez-Muros *et al*., 2014). The most widely studied insect species in terms of its potential to be used in animal feed is the *Hermetia illucens* larvae (Black soldier fly larvae or BSF LARVAE), *Musca domestica* larvae (common house fly larvae), *Tenebrio molitor (*mealworms*),* crickets and grasshoppers (EFSA Scientific Committee, 2015). The ability of Diptera species’ larvae to turn low-grade bio-waste into high-quality protein sources has made them a popular topic to explore in recent years, and major research foci have been on BSF larvae.

2.2 Nutritional composition of insects

Insects are a natural protein source of free-range poultry and can possibly become a sustainable alternative protein source. Most insect larvae or pupae are rich in proteins and typically consists of 40-70% crude protein (Rumpold & Schlüter, 2013a; Al-qazzaz, 2016). The fat, protein and energy content of insects varies depending on the insect species. Variation within species also exists depending on its rearing substrate, stage of development and environmental factors (Nguyen *et al.*, 2015; Veldkamp & Bosch, 2015). Sánchez-Muros *et al.* (2014) and Rumpold & Schlüter (2013b) compiled the nutrient composition of 150 and 236 edible insects, respectively. Their reviews included various insect species such as cockroaches, beetles, fly larvae, ants, termites, moths, etc. Most of the insect species in the study of Rumpold & Schlüter (2013b) contained high amounts of lysine (40-80mg/g protein), leucine (50-100 mg/g protein), methionine + cysteine (20-40 mg/g protein), and phenylalanine + tyrosine (60-120 mg/g protein).The mineral content of insects differs widely, with the exception of fly larvae, most insect species are relatively low in calcium (Rumpold & Schlüter, 2013a). Most insect species analysed had high levels of phosphorous, whereas only termites and crickets contain high levels of iron. Even though 100g of edible insects lack sufficient amounts of calcium, it has the potential to provide certain micronutrients such as zinc, copper, magnesium, selenium and iron (Banjo *et al.*, 2006; Rumpold & Schlüter, 2013a; b). However, it should be noted that insects have the ability to accumulate minerals from their rearing substrate within their body; therefore, calcium and other mineral concentrations can be manipulated (Klasing *et al.*, 2000).

Most of the nutrient compositions of insects specified in the above-mentioned research were derived from insects collected in the wild. If organic waste is utilised for the industrial farming of insects, the resulting nutrient profile of the insects ought to be considered to determine, amongst others, their suitability for animal feed or human food (Bessa *et al.*, 2020). In addition to the nutrient profile, the presence of potentially harmful ingredients such as allergens should be investigated to ensure safe feedstuff (Bessa *et al.*, 2017).

2.3 Black soldier fly larvae

Black soldier flies are polysaprophagous and synanthropic flies (Marshall *et al.*, 2015). In other words, they feed on decaying organic matter, are non-domesticated species, but still live close to human populations to benefit from them. Even though these flies can be found in abundance in every zoogeographic region of the world and naturally occur close to livestock manure piles, they are native to the Neotropics. Still, they have spread to other warm temperate parts of the world that offer them a suitable habitat (Marshall *et al.*, 2015). As a matter of fact, specimens from this Diptera species have been collected in South Africa from as early as 1915 (Marshall *et al.*, 2015). Since BSF larvae can easily be reared on decomposing materials such as human waste (restaurant and supermarket waste) (Spranghers *et al.*, 2017), manure (Newton *et al.*, 2005) and agricultural by-products (De Marco *et al.*, 2015; Meneguz *et al.*, 2018); these larvae have become popular from a waste management and feed perspective. It is now widely mass-reared for commercial purposes in South Africa, Europe, United States, Europe, and Asia.

2.3.1 Nutritional composition of black soldier fly larvae

Several research papers have reported the nutritional composition of BSF larvae meal (St-Hilaire *et al.*, 2007; De Marco *et al.*, 2015; Nguyen *et al.*, 2015; Maurer *et al.*, 2016; Barragan-Fonseca *et al.*, 2017). The protein content of the larvae typically ranges between 36% to 60% on a dry matter basis, whereas fat content can range between 26% - 55% (Table 2.1). Black soldier fly larvae meal is considered not only as a good protein source but also has a high AMEn content of ± 16.6 MJ/kg dry matter when fed to broilers (de Marco *et al.*, 2015). As mentioned, age of harvesting and the substrate used to rear the larvae can influence the nutritional composition of the larvae meal. Table 2.1 and Table 2.2 illustrate how different rearing substrates can influence larval nutritional composition. Table 2.3 reports the same information as Table 2.2, but the amino acid composition is expressed as a percentage of protein.

Processing methods such as different drying methods, cooking and drying temperatures as well as defatting of the larvae meal can also influence the nutritional composition and digestibility of the macro and micronutrients (Schiavone *et al.*, 2017b; Huang *et al.*, 2018). The total tract apparent digestibility coefficients for crude protein were reported to be is 0.51 for full-fat BSF larvae meal that had a crude fat content of 34.3% when fed to broiler in their finisher period (de Marco *et al.*, 2015). Black soldier fly larvae meal is a valuable source of digestible amino acids. De Marco *et al.* (2015) reported an apparent ileal digestibility coefficients (AIDC) of the amino acids in broilers ranging from 0.42 to 0.86, with a mean of 0.68 for all the amino acids in full-fat BSF larvae meal. The digestibility of amino acids in BSF larvae meal can increase as the fat content of the larvae meal decreases. Schiavone *et al.* (2017) reported a mean AIDC of 0.8 when BSF larvae meal was partially defatted (crude fat = 18%), whereas the AIDC for most of the amino acids increased when the larvae meal was highly defatted (crude fat = 4.6%). Insects generally contain adequate amounts of mono- and polyunsaturated fatty acids, which is comparable to that of fish and poultry (Rumpold & Schlüter, 2013b) whilst the diet the BSF larvae were reared on is known to influence the BSF larvae fatty acid profile (St-Hilaire *et al.*, 2007; Spranghers *et al.*, 2017; Cullere *et al.*, 2019). The predominant fatty acid in the fat of BSF larvae is lauric acid (Spranghers *et al.*, 2017), which hold several benefits for animals (Lieberman *et al.*, 2006). The lauric acid content in the lipids of BSF prepupae can reach up to 60%, especially if they are reared on starchy diets (Spranghers *et al.*, 2017). However, if desired, the fatty acid content can be manipulated. For example, Barroso *et al.* (2017) and St-Hilaire *et al.* (2007) demonstrated that the omega-3 (n-3) fatty acids content of larvae can be manipulated through the addition of fishmeal or fish offal to the substrate, subsequently lowering the n-6:n-3 ratio. Interestingly, the lauric acid content of BSF larvae reared on fish offal with manure increased together with the n-3 fatty acids, resulting in an even more beneficial fatty acid composition (St-Hilaire *et al.*, 2007).

2.3.2 Black soldier fly larvae meal in poultry diets

In recent years, attention has been given on the use of BSF larvae meal in poultry diets. Numerous research papers have been published on the use of BSF larvae meal as a protein source in the diets of broilers (Uushona, 2015; Schiavone *et al.*, 2017a; Brede *et al.*, 2018; Dabbou *et al.*, 2018; Nery *et al.*, 2018; Onsongo *et al.*, 2018; Pieterse *et al.*, 2019), laying hens (Maurer *et al.*, 2016; Borrelli

et al., 2017; Marono *et al.*, 2017; Cutrignelli *et al.*, 2018; Mwaniki *et al.*, 2018; Ruhnke *et al.*, 2018; Kawasaki *et al.*, 2019), quails (Widjastuti *et al.*, 2014; Cullere *et al.*, 2018; Mbhele *et al.*, 2019), turkeys, (Veldkamp *et al.*, 2012), ducks (Gariglio *et al.*, 2019a), guinea fowls (Wallace *et al.*, 2017, 2018), and pheasants (Loponte *et al.*, 2017).

Black soldier fly larvae meal can partially replace soya bean meal and fishmeal in broiler diets without adverse effects on production parameters. The replacement of 4% fishmeal with BSF larvae meal in broiler diets did not affect any of the production parameters, but a significant increase in carcass dressing percentage was recorded (Mohammed *et al.*, 2017). Even small inclusions of BSF larvae meal can a have a positive effect on production parameters since an improvement in growth and feed conversion ratio (FCR) in broilers were recorded with an inclusion rate as low as 0.5% and 1% (Choi *et al.*, 2018). The inclusion rate of BSF larvae meal can influence its effect on animal growth. Dabbou *et al.*, (2018) observed a positive response in production parameters (live weight, average daily gain and FCR) when partially defatted BSF larvae meal was added in broiler diets up to a 10% inclusion level. Then again, a 15% inclusion rate had a slightly negative effect on these parameters. A similar result was observed in turbot and catfish, when inclusions of BSF larvae meal was higher than 17% in turbot diets, a negative effect on FCR was recorded (Kroeckel *et al.*, 2012). When 13% - 49% of the fishmeal in catfish diets was replaced with BSF larvae meal, it had a positive effect on growth rate, but an 85% and 100% replacement had a negative effect on growth and FCR (Xiao *et al.*, 2018). This phenomenon can be because of the effect of chitin on nutrient digestibility. When diets of salmon and cod contained chitin levels of 1% or higher; a negative effect on protein digestibility, lipid digestibility, and growth rate was reported (Karlsen *et al.*, 2017). Similarly, high inclusions of chitin had a negative effect on the overall growth of blue crabs (Allman *et al.*, 2017). Additionally, Hansen & Karle, (1977) discovered that the adverse impact of krill meal on amino acid digestibility could be reduced by reducing the chitin content in the krill meal.

A factor to consider when comparing results between dietary larvae meal studies is the amino acid profile of the treatment diets. Some studies only substitute a protein source with larvae meal in monogastric animal diets (Oluokun, 2000; Elwert *et al.*, 2011). Even though iso-nitrogenous diets are used in these studies, a set amount of synthetic amino acids is often included in the treatment diets, without balancing the amino acid ratios or bearing in mind the ideal amino acid profile suitable for poultry diets. When 10% larvae meal is included in diets by merely replacing fishmeal or soya bean meal, various amino acids and minerals can be over or undersupplied when not taking the full nutritional profile of larvae meal vs soya bean meal or fishmeal into consideration. This may explain production differences between studies. For example, only very few studies balance arginine levels in their treatment diets, and previous research shows that a 0.2% difference in arginine can result in significant differences in broiler growth, FCR and well as lymphoid organ development (Kwak *et al.*, 1999). Another factor to consider when comparing results from studies is the fact that some studies use the total nitrogen or protein content in insect meal when balancing for an iso-nitrogenous diet, whereas other studies use crude protein values that have been corrected for the nitrogen content in chitin (Woods *et al.*, 2020).

It is important that BSF larvae should be included in the formulation and mixed within the diet, especially when fed to layer hens. For example, when BSF larvae was offered to laying hens separately on an *ad-lib* basis next to their formulated diet, no difference in performance was observed. Still, egg quality deteriorated (Ruhnke *et al.*, 2018). An improvement in FCR was recorded when soya bean meal in the diets of laying hens was replaced with 17% BSF larvae meal in the diets of laying hens, but a decrease in egg weight was observed (Marono *et al.*, 2017). In contrast, Bovera *et al.* (2018) observed a positive effect on egg mass and lay percentage with a 7% BSF meal inclusion. A 15% inclusion slightly decreased the ileal digestibility coefficient for macronutrients compared to the 7% inclusion rate. However; the egg weight, feed intake, and feed conversion rate were similar for hens in the 7%, 15% or control treatment (Bovera *et al.*, 2018). Similarly, Hopley (2015) also reported and an improved FCR and egg weights in hens receiving 10% BSF larvae meal.

2.4 *Musca domestica* fly larvae

2.4.1 Nutritional composition of *Musca domestica* (common house fly) larvae

Dried *M. domestica* larvae meal has high protein levels (>45%) and is rich in essential amino acids such as lysine and methionine (Odesanya *et al.*, 2011; Pieterse & Pretorius, 2014; Li *et al.*, 2017). *Musca domestica* larvae reared on broiler manure had lysine and methionine levels of 45 and 17 g/kg, respectively, whereas fishmeal has slightly higher levels of 57 and 23 g/kg, respectively (Hall *et al.*, 2018). Since methionine and lysine are the first and second limiting amino acids in traditional maize-soya-based poultry diets, the inclusion of *M. domestica* larvae meal to these diets should aid in balancing the diet in terms of lysine and methionine. Crude protein levels in *M. domestica* larvae can be as high as 60%, with a total tract protein digestibility of 69% when a 50% inclusion rate is used (Pieterse & Pretorius, 2014a). Hall *et al.* (2018) reported *M. domestica* larvae meal to have similar true ileal and apparent ileal digestibility coefficients as fishmeal. When 6% *M. domestica* larvae meal were included in broiler diets, there was no adverse effect on protein and fat digestibility (Khan *et al.*, 2018).

2.4.2 *Musca domestica* larvae meal in poultry diets

Feeding broilers live *M. domestica* larvae in addition to fishmeal-free diets improved weight gain when compared to chickens receiving diets including only fishmeal (Dordević *et al.*, 2008). *Musca domestica* larvae meal successfully replaced 5% fishmeal in broilers diets without any adverse effects on production parameters (Dordević *et al.*, 2008). Likewise, broilers receiving diets with 10% *M. domestica* larvae meal, performed comparably to broilers receiving diets with 10% fishmeal (Pretorius, 2011). Then again, inclusion rates higher than 25% larvae meal had a negative effect on FCR (Pretorius, 2011). It should be noted that in the latter trial, there was an oversupply of protein in the 25% larvae diets, therefore it is possible that the over-supply of protein rather than the over-supply of insect meal could have caused the negative effect on FCR. However, a similar outcome was obtained in fish. *Musca domestica* larvae meal could be added to diets of Nile Tilapia up to an inclusion rate of

27% without having a negative impact on production parameters, but higher levels decreased weight gain and FCR (Li *et al.*, 2017).

2.5 *Chrysomya chloropyga* larvae

Abattoir waste was identified as being one of the most problematic food waste types to manage in South Africa due to the hazardous nature of the waste type and its potential impacts on the environment and human health. An estimated 76102 tonnes of abattoir waste were produced in the Western Cape in 2015/2016 alone (Western Cape.gov 2017). Even though BSF larvae meal has proven to be effective in breaking down organic matter from waste streams, the waste conversion is severely affected when BSF larvae are reared on animal offal alone (Nguyen *et al.*, 2015). Therefore, there is a need to explore carrion Diptera species for offal waste recycling that can ultimately be used as a protein source in animal feed.

2.5.1 Nutritional composition of *Chrysomya chloropyga*

Parry (2017) investigated the effect of different substrates (kitchen waste, abattoir waste, swine manure) on the nutrient composition of three different carrion species: *Chrysomya chloropyga* (CC), *Chrysomya megacephala* and *Chrysomya putoria.* The protein content of *CC* was the highest when reared on swine offal, whereas the protein content of the other species was highest when reared on kitchen waste. The offal-reared CC larvae had the highest protein content compared to the other species reared on either of the substrates. The reported protein content of CC meal ranges between 48% - 58% on a DM basis whereas the fat content can range between 5% - 21% (Haasbroek, 2016; van der Merwe, 2018; van Aswegen, 2019).

2.5.2 *Chrysomya chloropyga* larvae meal in poultry diets

Research on the use of blowflies in animal diets is scarce. Gawaad & Brune, (1979) used a mixture of blow fly (*Phormia terraenovae*) and *M. domestica* larvae meal in the diets of broilers. The amino acid composition of the larvae meal was similar to fishmeal and soya bean meal. By using iso-nitrogenous diets in the trial, there were no significant differences for weight gain, FCR or carcass composition. Sing *et al.* (2014) evaluated the use of blowfly (*C. megacephala*) larvae meal in the diets of juvenile red tilapia. Not only did the blow fly larvae meal contain all the essential amino acids needed by the tilapia and had similar protein levels compared to fishmeal, but the complete replacements of fishmeal with blow fly larvae meal increased the growth rate and improved the FCR of the fish.

Chrysomya chloropyga is a blow fly native to Africa. Their larvae are carrion feeders, making them excellent in converting animal offal (Parry, 2017). Two studies investigated the use of CC meal, in the diets of broilers (van der Merwe, 2018; van Aswegen, 2019). Van der Merwe (2018) reported a higher slaughter weight and an increase in average daily gain when 10% CC meal were added to broiler diets. Even though a 15% inclusion did not improve growth parameters, broilers in this group performed similarly to the control group. Not only does CC larvae break down and consume otherwise inedible protein sources, but they also store available iron in their bodies which can ultimately replace the need

for dietary iron supplements in broiler diets (van Aswegen, 2019). Van Aswegen (2019) concluded that the use of CC meal in broiler diets showed no potential risk to the animal, had no adverse effect on carcass characteristics and had a positive effect on growth parameters.

Table 2.1 Chemical composition (% of dry weight) of *Hermetia illucens* and *Chrysomya chloropyga* larvae meal reared on different substrates

Protein source	Rearing substrate	Crude protein	Crude fat	Crude fiber or chitin	Ash	Reference
Full fat *H. illucens* prepupae meal	Kitchen waste	43.9	29.4	21.3 (crude fiber)	13.2	(Onsongo *et al.*, 2018)
	Chicken feed	41.2	33.6	6.2 (chitin)	10.0	(Spranghers *et al.*, 2017)
	Vegetable waste	39.9	37.1	5.7 (chitin)	9.6	(Spranghers *et al.*, 2017)
	Restaurant waste	43.1	38.6	6.7 (chitin)	2.7	(Spranghers *et al.*, 2017)
	Fruit	30.7	40.7	5.6 (chitin)	7.2	(Meneguz *et al.* 2018)
	Fruit & vegetables (30:70)	41.8	26.2	6.2 (chitin)	12.9	(Meneguz *et al.* 2018)
	Brewery-by-product	53.0	30.0	1.4 (chitin)	7.3	(Meneguz *et al.* 2018)
	Winery by-product	34.4	32.2	5.3 (chitin)	14.6	(Meneguz *et al.* 2018)
	Pig liver	47.0	18.7	-- [1]	--	(Nguyen *et al.*, 2015)
	Fish rendering	41.6	24.9	--	--	(Nguyen *et al.*, 2015)
	Fruit	31.5	55.3	5.2	--	(Jucker *et al.* 2017)
	Vegetables	60.1	8.7	13	--	(Jucker *et al.* 2017)
	Fruit & Vegetables	50.0	33.3	8.33	--	(Jucker *et al.* 2017)
	Wheat diet	40.0	33.8	--	5.1	(Liland *et al.*, 2017)
	Wheat Diet & seaweed (50:50)	33.7	22.2	--	10.5	(Liland *et al.*, 2017)
	Cereal-by products	36.9	34.3	--	--	(de Marco *et al.*, 2015)
Partially defatted *H. illucens* larvae meal	Cereal-by-products	55.3	18	5 (chitin)	--	(Schiavone *et al.*, 2017)
Full fat *C. chloropyga* (CC) larvae meal	Abattoir waste	58.6	20.99	23.8 (NDF)	12.97	(Haasbroek, 2016)
	Abattoir waste	56.3	8.7	5.3	4.28	(van Aswegen, 2019)

[1]: not stated

Table 2.2 Amino acid composition expressed as % dry weight of *Hermetia illucens* larvae meal (reared on different substrates), *Chrysomya chloropyga* larvae meal, fishmeal, and soya bean meal

Protein source	Rearing substrate	Crude protein %	ARG	HIS	ILE	LEU	LYS	MET	PHE	THR	TRP	VAL	Reference
Full fat BSF prepupae meal	Kitchen waste	43.9	2.11	1.35	1.77	2.78	2.81	0.8	1.64	1.63	NS	2.5	(Onsongo *et al.*, 2018)
	Chicken feed	41.2	2.03	1.36	1.72	2.86	2.34	0.76	1.7	1.64	0.67	2.41	(Spranghers *et al.*, 2017)
	Vegetable waste	39.9	2	1.24	1.73	2.8	2.26	0.76	1.63	1.54	0.58	2.48	(Spranghers *et al.*, 2017)
	Restaurant waste	43.1	1.99	1.38	1.91	3.06	2.3	0.71	1.64	1.62	0.54	2.82	(Spranghers *et al.*, 2017)
	Abattoir waste	44.20	2.19	1.56	2.06	3.02	2.83	0.78	1.59	1.79	0.74	2.79	(Lalander *et al.*, 2019)
	Dog food	42.80	2.12	1.08	2.04	3.43	2.64	0.77	1.77	1.63	0.58	2.69	(Lalander *et al.*, 2019)
	Human faeces	39.10	1.99	1.29	1.83	2.74	2.41	7.23	1.73	1.47	0.66	2.58	(Lalander *et al.*, 2019)
	Poultry manure	41.60	2.05	1.29	1.71	27.50	2.94	0.85	1.75	1.54	0.71	2.48	(Lalander *et al.*, 2019)
	Wheat diet	40.00	1.80	1.12	1.56	2.56	2.36	0.68	1.60	1.56	--[1]	2.32	(Liland *et al.*, 2017)
	Wheat diet + seaweed (50:50)	33.70	1.55	0.81	1.35	2.26	1.89	0.47	1.15	1.35	--	1.92	(Liland *et al.*, 2017)
	Cereal-by-products	36.9	1.94	1.13	1.72	2.4	2.23	0.91	1.44	1.52	--	2.2	(de Marco *et al.*, 2015)
Partially defatted BSF larvae meal	Cereal-by-products	55.3	2.15	1.23	1.85	2.86	2.12	0.64	1.66	1.72	--	2.72	(Schiavone *et al.*, 2017)
***C. chloropyga* (CC) larvae meal**	Animal offal	58.58	2.24	1.07	1.3	1.3	1.96	0.62	1.99	1.23	--	1.67	(Haasbroek, 2016)
Fishmeal as reference	N/A	72	4.21	1.74	3.23	5.46	5.47	2.16	2.87	3.07	0.83	3.90	(NRC, 1994)
Soya bean meal as reference	N/A	47.5	3.48	1.28	2.12	3.74	2.96	0.67	2.34	1.87	0.74	2.22	(NRC, 1994)

[1]: not stated

Table 2.3: Amino acid composition expressed as % of protein of BSF larvae meal (reared on different substrates), *Chrysomya chloropyga* larvae meal, fishmeal, and soya bean meal

Protein source	Rearing substrate	Crude protein %	ARG	HIS	ILE	LEU	LYS	MET	PHE	THR	TRP	VAL	Reference
Full fat BSF prepupae meal	Kitchen waste	43.90	4.81	3.08	4.03	6.33	6.40	1.82	3.74	3.71	NS	5.69	(Onsongo *et al*., 2018)
	Chicken feed	41.20	4.93	3.30	4.17	6.94	5.68	1.84	4.13	3.98	1.63	5.85	(Spranghers *et al*., 2017)
	Vegetable waste	39.90	5.01	3.11	4.34	7.02	5.66	1.90	4.09	3.86	1.45	6.22	(Spranghers *et al*., 2017)
	Restaurant waste	43.10	4.62	3.20	4.43	7.10	5.34	1.65	3.81	3.76	1.25	6.54	(Spranghers *et al*., 2017)
	Abattoir waste	44.20	4.96	3.53	4.67	6.84	6.40	1.77	3.59	4.05	1.67	6.32	(Lalander *et al*., 2019)
	Dog food	42.80	4.95	2.53	4.76	8.01	6.17	1.81	4.14	3.80	1.36	6.29	(Lalander *et al*., 2019)
	Human faeces	39.10	5.10	3.30	4.67	7.01	6.17	18.50	4.42	3.76	1.69	6.59	(Lalander *et al*., 2019)
	Poultry manure	41.60	4.92	3.09	4.10	66.10	7.07	2.05	4.20	3.70	1.71	5.97	(Lalander *et al*., 2019)
	Wheat diet	40.00	4.50	2.80	3.90	6.40	5.90	1.70	4.00	3.90	--	5.80	(Liland *et al*., 2017)
	Wheat diet + seaweed (50:50)	33.7	4.60	2.40	4.00	6.70	5.60	1.40	3.40	4.00	--	5.70	(Liland *et al*., 2017)
	Cereal-by-products	36.90	5.26	3.06	4.66	6.50	6.04	2.47	3.90	4.12	--	5.96	(de Marco *et al*., 2015)
Partially defatted BSF larvae meal	Cereal-by-products	55.30	3.89	2.22	3.35	5.17	3.83	1.16	3.00	3.11	--	4.92	(Schiavone *et al*., 2017)
***C. chloropyga* (CC) larvae meal**	Animal offal	58.58	3.82	1.83	2.22	2.22	3.35	1.06	3.40	2.10	--	2.85	(Haasbroek, 2016)
Fishmeal as reference	N/A	72.00	5.85	2.42	4.49	7.58	7.60	3.00	3.99	4.26	1.15	5.42	(NRC, 1994)
Soya bean meal as reference	N/A	47.50	7.33	2.69	4.46	7.87	6.23	1.41	4.93	3.94	1.56	4.67	(NRC, 1994)

[1]: not stated

2.6 The effect of insect meal and insect compounds on animal health

2.6.1 Basics of the immune system

The immune system of an animal is a complex system that shields the animal against pathogens responsible for infections or diseases. The immune system can be divided into two arms, namely the adaptive (acquired) and innate immune (non-specific) immune system. Both systems recognise foreign particles, but they trigger different cellular and molecular mechanisms when eliminating an antigen (Fellah *et al.*, 2008; Reyes-Cerpa *et al.*, 2012). The way these two systems recognise and respond to pathogens distinguishes them from one another. The innate immune system is responsible for the first line of defence when a foreign organism enters the body (Babu & Raybourne, 2008; Riera Romo *et al.*, 2016). No previous exposure to a pathogen or antigen is needed for the innate immune system to be activated. The innate immunity includes barriers such as the skin or mucous membranes. The innate immunity encompasses the compliment system, phagocytic cells (macrophages and polymorphonuclear leukocytes), inflammatory cells, and all of the antimicrobial substances in the blood and lymph (Babu & Raybourne, 2008) This system relies on pattern-recognition receptors (PRRs). These receptors recognise molecules that are typically associated with pathogens. These molecules include polysaccharides, peptidoglycans, nucleic acids and lipoproteins (Iwasaki & Medzhitov, 2015). These receptors are standard and only recognise antigen patterns.

Acquired or specific immunity, on the other hand, is activated because of cellular memory of previous exposure to antigens or pathogens. There are two lymphocytes, namely B and T lymphocytes, that controls the acquired immune response. These lymphocytes also recognise antigens *via* receptors (Babu & Raybourne, 2008; Fellah *et al.*, 2008; Schijns *et al.*, 2014).The immunity acquired through the B lymphocytes is referred to as the humoral immune response and involves the production of antibodies. In mammals, the B lymphocytes mature in the bone marrow, whereas in birds, it matures in the bursa of Fabricius. The T lymphocytes are responsible for cell-mediated immunity. The T lymphocytes either kill the infected host cells directly or produce cytokines and activate other immune cells to assist in eradicating invading pathogens (Babu & Raybourne, 2008; Fellah *et al.*, 2008; Schijns *et al.*, 2014). To prevent the colonisation of a pathogenic microorganism after entering a host's body, the animal's immune system comprises of various defence peptides such as lysozymes, complement factors, antibodies, and other lytic factors. These factors form the first line of defence and contribute towards the prevention of infectious diseases.

2.6.2 Effect of insect meal and insect compounds on the innate immune response

2.6.2.1 Effect on serum lysozyme activity

Lysozymes are one of the important defence mechanisms of the non-specific humoral immune system and can be used as a marker of the non-specific immune response against pathogens (Saurabh & Sahoo, 2008). Phagocytes in the animals' blood secrete lysozymes. These lysozymes are capable of disrupting the cell walls of bacteria through hydrolysis of the mucopeptide in the cell walls of Gram-positive bacteria, leading to lysis of the bacterial cell (Chassy & Giuffrida, 1980). Lysozymes are not only capable of destroying bacterial

cells on their own, but they also aid in activating the compliment, a system composed of serum proteins that react against pathogens through a molecular cascade, resulting in microbial lysis (Wardlaw, 1961; Saurabh & Sahoo, 2008). Furthermore, certain antibodies such as IgA are only effective in lysing *Escherichia coli* in the presence of both lysozyme and the complement system (Adinolfi *et al.*, 1966).

It is possible that chitin in the insect cuticle, and several bioactive compounds in insects, can induce lysozyme activity in animals. Shanthi Mari *et al.* (2014) reported an increase in lysozyme and phagocytic activity in the blood of bacterially infected carp due to chitin supplementation. Similarly, the addition of 40% cricket meal in catfish diets almost triplet the lysozyme activity by increasing the levels in the blood from 8.5 U/ml to 22.2 U/ml (Taufek *et al.*, 2018). Then again, adding different levels of BSF larvae meal in yellow catfish diets had no significant effect on lysozyme activity (Xiao *et al.*, 2018). Likewise, no differences in serum lysozyme activity were observed when 25% or 50% of the fishmeal in trout diets were replaced with mealworm meal (Henry *et al.*, 2018a). However, even though the inclusion of 9%, 18%, or 27% mealworm in diets of healthy yellow catfish had no effect on the plasma lysozyme concentrations, when these catfish were challenged with *Pelteobagrus fulvidraco,* the lysozyme concentrations significantly increased in fish receiving dietary mealworms (Su *et al.*, 2017). Considering the results in the studies mentioned above, it is possible that insect meal has the greatest effect on lysozyme concentration when the animal is infected with a pathogen.

2.6.2.2 Effect on serum bactericidal activity

Together with lysozymes, the blood of the host contains cellular components with phagocytic abilities that kill the bacteria it encounters. Blood (serum) also contains soluble elements that protect the host from invading pathogens (Riera Romo *et al.*, 2016). Therefore, the serum of the animal exhibits bactericidal activity by means of several elements. In addition, the complement system can initiate an enzymatic cascade capable of lysing targeted bacterial cells (Nordahl *et al.*, 2004). Acute-phase proteins, such as mannose-binding proteins, also exhibit bactericidal competence by attacking bacterial cell walls (Fernie-King *et al.*, 2002; Al-Khalifa, 2016).

Feeding mealworms (*Tenebrio molitor*) to sea bass did not affect serum bacteriolytic activity against Gram-negative *E. coli* (Henry *et al.*, 2018b). In contrast, dietary mealworms resulted in the speedier killing of *E. coli* cells in the serum of rainbow trout (Henry *et al.*, 2018a). The effect of dietary insects on serum bactericidal activity against Gram-negative bacteria is scarce. Since there are only a few studies on the serum bactericidal effect of dietary insects on Gram-negative bacteria, it is unclear if all insect meals will exert this activity in different animals.

Certain insect meals can reduce the pathogen load in the organs of the host. The bacterial load in the intestines of *Aeromonas hydrophila* infected catfish reduced from 58 x 10^7 CFU/g to 3.2 x 10^7 CFU/g when receiving 35% cricket meal. Likewise, *A. hydrophila* levels in the liver decreased from 0.625 CFU/g to 0.134 CFU/g when receiving cricket meal (Taufek *et al.*, 2018). Chitin might be involved in the activities mentioned above. Even though dietary chitin had no effect on lysozyme activity in gilthead seabreams, it increased the activity of the alternative complement pathway, one of three pathways that opsonise and kills pathogens in serum (Esteban *et al.*, 2001).

2.6.2.3 Impact on the anti-parasitic activity

It is possible that the intake of insects stimulates an immune response and increase the anti-parasitic activity in animals due to similarities in the composition of insects and the exoskeleton of parasites (Henry *et al.*, 2018b). Pathogens use several mechanisms that help them bypass the immune system of animals. One of their mechanisms is to produce protease that attacks and invades the complement system (Potempa & Potempa, 2013). An increase in trypsin inhibition activity in the serum represents the capacity of the animal to counteract the release of protease by the pathogen. Dietary mealworms in the diets of sea bass and rainbow trout increased the trypsin inhibition activity of their serum (Henry *et al.*, 2018a; Henry *et al.*, 2018b). Since antiprotease activity (serum trypsin inhibition) is usually correlated with anti-parasitic activity (Henry *et al.*, 2015), it can be alleged that mealworms express anti-parasitic activity in fish.

2.6.2.4 Effect on macrophages and cytokines

An innate immune response is initiated when Toll-like receptors recognise pathogens. Subsequently, this will lead to the induction of various cytokines and production of nitric oxide (NO), which in turn is a significant mediator of immunostimulatory activity (Ohta *et al.*, 2014). An acidic polysaccharide, namely dipterose, was identified and extracted from the pupae of melon flies. Dipterose triggered macrophages to produce NO levels similar to lipopolysaccharide, a widely known and potent immunostimulant (Ohta *et al.*, 2014). Dipterose-BSF can activate the innate immune response by stimulating the induction of various cytokines and macrophages via the TLR signalling pathway (Ali *et al.*, 2019). Similarly, a polysaccharide extracted from the silkmoth (*Antheraea yamamai*) activates the mammalian innate immune response through activation of macrophages (Ohta *et al.*, 2016).

Chitin in the exoskeleton of insects also exhibits immunomodulatory properties. The immunostimulatory effect of chitin and its derivates has been explored since the 1980s. It was demonstrated that chitin and its derivates could activate natural killer cells, as well as peritoneal macrophages. These cells express pro-inflammatory cytokines such as interferon-gamma (IFN-γ), interleukin-1β (IL-1β) and colony-stimulating factors (Nishimura *et al.*, 1984; Lida *et al.*, 1987). Interferon-gamma further primes macrophages and enhances their oxidative burst (Shibata *et al.*, 1997) and is also involved in resistance to *Salmonella* infection (Lalmanach & Lantier, 1999). Nonetheless, it should be noted that the composition of the chitin mixtures has a vast effect on its immunostimulatory properties. For example, a combination of 30% chitin and 70% chitosan had a more significant impact on macrophage activation compared to either pure chitin or pure chitosan (Nishimura *et al.*, 1984)

2.6.2.5 Effect on anti-inflammatory responses

Controlled inflammation is a normal and important defence mechanism of the immune system. It protects the animal against infections since it initiates the destruction of pathogens and starts the repair process of damaged tissue. Many cell types and chemical mediators are involved in the inflammatory response, and when controlled properly, inflammation is essential in maintaining homeostasis to remain healthy (Calder, 2011). However, pathological inflammation involves the loss of tolerance and regulatory

processes. Irreparable damage to the host tissue can occur when pathological inflammation becomes excessive (Calder, 2009, 2011). The inflammatory response involves the conversion of arachidonic acid to pro-inflammatory cytokines (Changxing *et al.*, 2018). Pro-inflammatory cytokines are essential in initiating an immune response and contribute towards generating an inflammatory response (Genovese *et al.*, 2013). However, inflammation is thought to be a factor of the immune response that interrupts growth-related physiology, resulting in impaired growth rate when an animal contracts a disease (Korver & Klasing, 1997). The balance between anti-inflammatory cytokines and pro-inflammatory cytokines is a factor that determines the characteristics of infections (Reyes-Cerpa *et al.*, 2012). Cytokine activity inhibition is sometimes observed during viral diseases, and when the expression of anti-inflammatory cytokines is reduced, chronic infection can occur (Reyes-Cerpa *et al.*, 2012). For this reason, dietary factors that promote anti-inflammatory factors are beneficial to maintain an equilibrium.

Henry *et al.* (2018) observed a substantial reduction in ceruloplasmin and myeloperoxidase activity in the serum of sea bass fed diets containing 25% mealworm meal. These responses are all associated with anti-inflammatory activity (Halliwell & Gutteridge, 1990; Dorward *et al.*, 2012; Hodgkinson *et al.*, 2015; Henry *et al.*, 2018). But more importantly, ceruloplasmin protects the animal against toxic oxygen metabolites released during the inflammatory process (Halliwell & Gutteridge, 1990). Furthermore, protein-enriched fractions extracted from *M. domestica* larvae inhibited multiple pro-inflammatory responses (Chu *et al.*, 2011). On the other hand, a bioactive polysaccharide (dipterose-BSF) extracted from BSF larvae enhanced the expression of pro-inflammatory cytokines and interferon-β in mouse macrophage cells (Ali *et al.*, 2019).

2.6.3 Effect of insect meal and insect compounds on the humoral immune response

Certain factors such as polyphenols and chitin that are present in insects (Janssen *et al*., 2019) are known to stimulate immunoglobulin production (Koide, 1998; Taira *et al*., 2015). Chitosan has the ability to increase immunoglobulin-G (IgG) levels and the humoral immune response in piglets and mice (Zaharoff *et al*., 2007; Li *et al*., 2013). Similarly, super mealworm (*Zophobas morio*) meal increased the immunoglobulin concentrations in *Salmonella* infected broilers (Islam & Yang, 2017). Black soldier fly larvae meal in the diets of weaner piglets did not affect serum IgG levels, but immunoglobulin-A (IgA) increased linearly and quadratically as inclusion rate increased from 1% - 4% (Yu *et al.*, 2020a). Additionally, a linear increase of IgA was found in the ileal mucosa of piglets that received 1, 2% or 4% BSF larvae meal in their diets (Yu *et al.*, 2020b). Then again, dietary mealworms had no influence on general immunoglobulin concentrations in unchallenged broilers (Jin *et al*., 2016) and BSF larvae oil in turkey diets did not affect IgM concentrations (Sypniewski *et al.*, 2020). Neither did the inclusion of 60% BSF larvae meal in salmon diets have any effect on antibody response after vaccination against an infectious virus (Li *et al.*, 2019). Insect meal may have the ability to influence the transcriptional levels of immune-related genes. The inclusion of mealworm meal in the diets of catfish increased the expression of immunoglobulin-M (IgM) in the liver, and transcriptional levels of genes for IgM increased in the spleen (Su *et al.*, 2017).

2.6.4 Effect of insect meal on antioxidation properties

As a result of various physiochemical conditions, pathological states and endogenous systems, free radicals are generated in human and animal bodies. The correct balance between antioxidants and free

radicals are essential for optimal physiological function and prevention of oxidative stress. Oxidation can cause lipids, proteins and DNA alteration that can ultimately trigger diseases (Lobo *et al.*, 2010). The intake of dietary antioxidants is thus essential.

There is an indication that insect meal could increase an animal's antioxidant defence system. Insect meal is high in tocopherols (Secci *et al.*, 2018) and selenium (Rumpold & Schlüter, 2013a), which are effective antioxidants in lipid systems. *In-vitro* studies indicated that BSF protein derivatives could protect animal cells against oxidative damage, whereas, fishmeal and chicken meal showed little or no anti-oxidative properties (Mouithys-Mickalad *et al.*, 2020). An increase in certain anti-oxidative enzymes and a decrease in lipid peroxidation have been detected in the intestines of mealworm-fed rainbow trout (Henry *et al.*, 2018a). The inclusion of mealworm meal in catfish diets decreased their plasma malondialdehyde (MDA) contents (Su *et al.*, 2017), which is the end product of lipid peroxidation that causes cell damage (Achuba & Osakwe, 2003). An increase in plasma superoxide dismutase (SOD) levels, which is involved in the elimination of free radicals, were also recorded in these mealworm-fed catfish (Su *et al.*, 2017). Likewise, Henry *et al.* (2018a) also reported an increase of SOD in mealworm-fed rainbow trout. Correspondingly, replacing 25% of fishmeal with BSF larvae meal increased serum SOD levels of yellow catfish (Xiao *et al.*, 2018).

Glutathione peroxidase serves as a major defence mechanism against oxidative damage by removing hydrogen peroxides from the body. Selenium is one of the most important dietary factors that can enhance its activity. In the same fashion as selenium, BSF larvae meal increased the glutathione peroxidase levels in the blood of broilers (Dabbou *et al.*, 2018). Since BSF and mealworm meal generates the same antioxidant responses as selenium and vitamin E (Su *et al.*, 2017; Dabbou *et al.*, 2018), it can be hypothesised that insect meal can serve as a dietary antioxidant.

2.6.5 Effect of insect meal on haematological parameters, biochemical parameters, and organ weights

Biochemical, as well as haematological profiles usually provide valuable information on the health status of animals. Several studies evaluated the effect of insect meal on blood parameters. The entire replacement of fishmeal with cricket meal significantly increased the white blood cells, total protein, and globulin levels in the blood of African catfish (Taufek *et al.*, 2018). Similarly, BSF larvae meal increased total protein and globulin levels in the serum of weaner piglets (Yu *et al.*, 2020a). On the other hand, BSF larvae meal in the diets of Muscovy ducks did not affect total serum protein count, suggesting a similar dietary protein availability from BSF larvae diets (Gariglio *et al.*, 2019b). The replacement of 4% fishmeal with 4% BSF larvae meal resulted in increased haemoglobin, packed cell volume, and red blood cells in broilers (Mohammed *et al.*, 2017). The chitin in the exoskeleton of the insects might be responsible for these changes since the dietary intake of 1% chitin significantly increased neutrophils, monocytes, lymphocytes, total white blood cells, red blood cells, and haematocrit values in carp (*Cirrhina mrigala*) (Shanthi Mari *et al.*, 2014).

In contrast with these findings, no effect on erythrocyte, leukocyte or leukocyte differential count was recorded in the blood of Muscovy ducks receiving 3% - 6% BSF larvae meal in their diets (Gariglio *et al.*, 2019b). Similarly, Dabbou *et al.* (2018) examined several haematological and serum parameters and found no major alterations on broilers that received partly defatted BSF larvae meal. The use of BSF larvae meal in the diets of guinea fowl also had no effect on any of the leucocyte differentials and most of the haematological parameters were unaffected by dietary BSF larvae meal, however, the MCH values

(amount of haemoglobin in red blood cells) increased compared to the control group (Wallace *et al.*, 2018). The biochemical parameters were also unaffected in the study mentioned above since serum lipid concentrations, metabolites, electrolytes, and enzymes in BSF larvae-fed guinea fowls were similar compared to the control group (Wallace *et al.*, 2018).

Even though biochemical and haematological profiles usually provides valuable information on the health status of animals, there is a lack of reference values for poultry, which restricts its use in this animal class (Talebi *et al.*, 2005). Certain immune organs such as the spleen and thymus are essential for the maintenance of a healthy immune system (Wallace *et al.*, 2018). Therefore, these lymphoid organ weights can be assessed and used as an indicator of the immune status of poultry. Dietary BSF larvae did not affect organ weights or lymphoid organ weights in broilers (Uushona, 2015) or guinea fowl (Wallace *et al.*, 2018). Similarly, replacing 50% of the soya bean meal in layer hen diets with BSF larvae meal had no significant effect on liver, spleen or crop weights (Bovera *et al.*, 2018).

2.6.6 Anti-viral activity of insect compounds

A few anti-viral mechanisms for insect protein extracts have been documented. Protein fractions extracted from *M. domestica* larvae had a direct deactivating effect on the pseudorabies virus (PRV) and hindered the penetration and absorption of the virus into cells (Yong Wang *et al.*, 2012). A compound extracted from the haemolymph of tobacco budworm (*Heliothis virescens*) larvae had anti-viral properties against human immunodeficiency virus-1 (HIV-1) and herpes simplex virus-1 (HSV-1) (Ourth, 2004). It was hypothesised that a peptide in the extraction binds and localise in the cytoplasmic side of the membrane. This action blocks the assembly and the exit of a virus from a host cell (Resh, 1999; Ourth, 2004). Alloferon, extracted from *Calliphora vicina* flies, also triggered intracellular responses that inhibit influenza-A and influenza-B virus reproduction in mice (Chernysh *et al.*, 2002).

The haemolymph of the giant silkworm (*Lonomia oblique*) exhibited potent anti-viral activity against measles, polio, and influenza viruses (Greco *et al.*, 2009). Even though the haemolymph of giant silkworms did not display virucidal activity when directly tested on viruses, it did result in much lower viral titers when cell cultures were exposed to the haemolymph before infection occurred. Hence it is believed that before the virus infection takes place, an intracellular mechanism of anti-viral action is initiated by the haemolymph that may inhibit some important mechanism for viral replication (Greco *et al.*, 2009)

2.6.7 Influence of insect meal on mortality rate of animals

Since mortality rate on a farm can have a severe effect on the profit margin in livestock production, it will be beneficial to have a dietary constitute that will decrease the mortality rate, especially in diseased animals. Interestingly, insect meal appears to reduce the occurrence of mortalities in diseased animals. After infection with a pathogenic bacterium, *Aeromonas hydrophila*, catfish that received 40% cricket meal had a mortality rate of 27%, whereas the mortality rate of catfish in the control group was 90% (Taufek *et al.*, 2018). Similarly, a 21% reduction in mortality rate was recorded when catfish received 27% mealworm meal (Su *et al.*, 2017). Furthermore, supplementing *Aphanomyces invadans* infected Mrigal carp fish with 1% chitin

resulted in a cascade of first-line immune responses that ultimately led to a 70% reduction in mortality rate (Shanthi Mari *et al.*, 2014).

2.6.8 Effect of insect meal on the gut microbiome and gut morphology of animals

Dietary supplementation with small amounts of insect meal can have a significant impact on the gut microbiome. Larvae meal diets can change the microbiota in chickens. Black soldier fly larvae meal increased the numbers of chitin degrading microorganisms, which correlates with an increase of short-chain fatty acids in the gut, which in turn promotes gut health (Borrelli *et al.*, 2017). Supplementation of chicken diets with either Turkestan cockroach meal, yellow mealworm meal, or BSF larvae meal, had effects on the microbiota counts of different parts of the gastrointestinal (GI) tract (Józefiak *et al.*, 2018). When considering the microbiota counts in the crops of chickens, mealworm meal decreased *Bacteroides–Prevotella* clusters. Turkestan cockroach meal increased the *Clostridium leptum* subgroup counts, whereas BSF larvae meal increased *Lactobacillus* spp counts and *Eubacterium rectale* clusters in the crop and caeca (Józefiak *et al.*, 2018). Many species of the *Clostridium leptum* subgroup, as well as the *E. rectale* cluster, are butyrate-producing microbiota (Barcenilla *et al.*, 2000; Lay *et al.*, 2005). Dietary BSF meal also increased butyrate content in the cecum of weaner piglets (Yu *et al.*, 2020b). Not only does butyrate play an essential role in maintaining the health of the large intestine (Lay *et al.*, 2005), it is also useful in reducing Salmonella counts in the ceca (Van Immerseel *et al.*, 2005).

High inclusion levels of insect meal can also negatively affect gut morphology. Dabbou *et al.* (2018) reported shorter villi and deeper crypts when broilers received 15% BSF larvae meal in their diets. Similarly, Biasato *et al.* (2017) observed shorter villi and deeper crypts in broilers fed 15% *T. molitor* (yellow mealworm) larvae meal. Shorter villi decrease the absorptive area of the intestinal epithelium and are an indication of sub-optimal digestive enzyme action and lowered transport of nutrients across the intestine. An increase in crypt depth reflects a shorter life span of the villi, and an increased need for renewal, which ultimately increases the need for energy for gut renewal (Dabbou *et al.,* 2018). Lower villus height and deeper crypts could lead to poor digestion and less absorption of nutrients, reflected by poor performance (Qaisrani *et al.*, 2014). Both Dabbou *et al.* (2018) and Biasato *et al.* (2017) reported poor performance in chickens receiving 15% inclusion of insect meal. Therefore, if a negative effect in growth is observed with when high inclusion levels of insect meal is used in a diet, it might be due to deteriorated gut morphology.

2.7 Antimicrobial substances in insects

Most multicellular organisms produce several antimicrobial peptides (AMPs) from different structural classes. Since insects don't produce lymphocytes and antibodies, they rely on broad-spectrum AMPs to protect them from bacteria, fungi, viruses and protozoa (Zasloff, 2002). The mode of action is not always clear, but AMPs such as defensins have almost immediate lytic effects on Gram-positive bacteria (Cociancich *et al.*, 1993). These peptides share key features such as low molecular weight (below 5kDa), a positive net charge at physiological pH and for most of them, amphilic α-helixes or hairpin-like β-sheets (Bulet *et al*., 1999). An insect usually produces a unique repertoire of AMPs with structural features that overlap and targets a specific microorganism. When several of the AMPs act in synergy, it provides the insect with a potent defence against pathogens (Bulet *et al.*, 1999).

The antimicrobial properties of insects have been explored for over a century. At the beginning of the 20th century, Nicholls (1912) discovered that pupae from larvae reared on material contaminated with *Staphylococcus aureus* were free of this bacterium after removal from the substrate. Glaser (1918) found that after introducing a bacterium to grasshoppers, the grasshopper obtained immunity against the bacterium since the blood of the grasshopper showed a degree of antagonism against the selected bacteria. In 1948, Frings *et al.* (1948) reported that the blood from the large milkweed bug expressed antibacterial properties against *S. aureus* and *Bacillus subtilis*. In the same year, Pavan (1948) extracted a new antibiotic, named Iridomyrmecin, from the Argentine ant.

The first antimicrobial peptide (cecropin) was purified in 1980 (Hultmark *et al.*, 1980). Since then, over 150 insect AMPs have been identified (Yi *et al.*, 2014). Antimicrobial peptides are small, biologically active molecular polypeptides produced by multicellular organisms and can be classified into four families based on their unique sequences or structure. For example, cecropin and moricin are α-helical peptides, insect defensin and *drosomycin* are cysteine-rich peptides, apidaecin and drosocin are proline-rich peptides, whereas attacin and gloverin are glycine-rich peptides (Yi *et al.*, 2014). Defensin and defensin-like antimicrobial peptides have been extracted from the following Diptera species: *Protophormia terraenovae* (blue bottle blow fly), *Sarcophaga peregrina* (flesh fly), *Stomoxys calcitrans* (stable fly), *Lucilia sericata* (common green bottle fly) and *Eristalis tenax* (common drone fly).

Antimicrobial peptides have broad-spectrum antimicrobial activity against bacteria, viruses, and fungi. An antibacterial substance isolated from larvae of the sawfly, *Acantholyda parki* S., showed antimicrobial activity against Gram-positive and Gram-negative bacteria. The substance was active against *E. Coli, Salmonella enteritidis, B. subtilis, Candida albicans KCTC, S. aureus,* and *Micrococcus luteus* (Leem *et al.*, 1999). An antimicrobial compound extracted from *M. domestica* larvae inhibited the growth of Gram-positive bacteria, *Bacillus thuringiensis;* and the yeast, *Saccharomyces cerevisiae*. Still, it did not affect the growth of the Gram-negative bacteria, *E. coli* (Meylaers *et al.*, 2004). Extracts from BSF larvae also exhibited antimicrobial properties. Park *et al.* (2014) reported low molecular weight antimicrobial factors extracted from BSF larvae to be effective against a broad range of microorganisms including Gram-positive bacteria (*Kocuria rhizophila*, *M. luteus*, *B. subtilis*), Gram-negative bacteria (*E. coli*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*) and fungi (*C. albicans*). Moreover, during the melanisation process of BSF larvae, a brown coloured substance is secreted. This cytotoxic phenolic biopolymer has antimicrobial properties and is involved in larval immunity (Sugumaran, 2002; Park *et al.*, 2014).

Insect AMPs has the potential to be used in agriculture and medicine. Some insect AMPs have been engineered into transgenic plants to protect the plants against pathogens (Ohshimax *et al.*, 1999; Mitsuhara *et al.*, 2000; Rahnamaeian *et al.*, 2009). Granted that insects' AMPs have antimicrobial activity against a broad range of microbes; and pathogens seldom develop resistance against these AMPs (Wang *et al.*, 2017), there is also the potential for the use of AMPs in livestock production as an alternative to antibiotics.

Contaminated poultry feed can lead to human illness, especially if the carcass gets contaminated with gut contents during the slaughtering process. One of the microbes that cause major concern in the poultry industry is *Salmonella spp*, which causes Salmonellosis in humans. Interestingly, AMPs extracted from house fly larvae that were exposed to *Salmonella* were almost as effective as artificial antibiotics to treat the *Salmonella pullorum*-infected chickens (Zhou *et al.*, 2014). Furthermore, the AMPs extracted from BSF larvae

immunised with *S. pullorum* did not only show antimicrobial activity against *S. pullorum in vitro*, it also increased the goblet cells and the lymphocyte cells in the intestines of *S. pullorum* infected chickens (Wang *et al*., 2017). This phenomenon can also aid in eliminating pathogens in the intestine since goblet cells assist in expelling bacteria by stimulating mucus secretion from crypt cells (Ridler, 2016). Additionally, goblet cells do not only protect the intestinal epithelium but together with lymphocyte cells plays an essential role in the gut immunity of young animals (Wang *et al.*, 2017).

Therefore, if AMPs usually found in insects are present and still active in dietary insect meal, it has the potential to decrease the pathogen load in the flock, resulting in a healthier flock and reduced chance of carcass contamination during the slaughtering process

2.8 Possible risks and constraints associated with insect meal production for feed

Consumer acceptance of this new feed source is essential. One factor that might influence their acceptance is the effect of dietary insect meal on the taste of the meat at the end. Published data indicates that the use of larvae meal in fish and poultry diets has minimal effect on sensory attributes of the meat. Pieterse *et al.* (2014) reported *M. domestica* fed chicken meat to have a prominent chicken aroma with a less noticeable chicken flavour and higher sustained juiciness. Even though larvae meal caused a slightly higher metallic aroma and aftertaste in the meat, it was considered unlikely to be detected by consumers. Correspondingly, Borgogno *et al.* (2017) reported BSF-fed rainbow trout meat to have an onset of metallic flavour, whereas the fish from the control group had more boiled fish algae flavours. Furthermore, the fibrousness of the trout meat decreased as the BSF levels in the diet increased. On the other hand, BSF larvae meal had no effect on the aroma, flavour, juiciness or tenderness of broiler chicken and quail meat with dietary inclusions rate up to 15% (Cullere *et al.*, 2018; Pieterse *et al.*, 2019).

It should be noted that certain insect species can contain antinutritional factors, toxins as well as allergens (Rumpold & Schlüter, 2013a; Bessa *et al.*, 2020). When insects are gathered from the wild, these insects might contain toxins from microorganisms such as botulinum toxin or aflatoxins which can cause diseases (Schabel, 2010). Insects harvested from pesticide-treated areas may also contain pesticides (Schabel, 2010). Insects, as well as BSF larvae, bioaccumulate heavy metals such as cadmium, lead and arsenic from their feedstock (Charlton *et al.*, 2015; Butt *et al.*, 2018; Cai *et al.*, 2018). The bioaccumulation of heavy metals, particularly cadmium, can pose a risk for the use of insects as animal feed (Charlton *et al.*, 2015).

Charlton *et al.* (2015) collected nine larvae meal samples from four fly species that were produced in different countries. The samples were tested for 1140 contaminants. The contaminants tested for includes pesticides, heavy metals, mycotoxins, dioxins, polychlorinated biphenyls, polyaromatic hydrocarbons, and veterinary medicines. *Musca domestica* and *C. Chloropyga* in this study were reared on manure. Black soldier fly larvae were reared on spent grain and fish feed waste, whereas pig offal was used to rear *Calliphora vomitoria*. Out of the 1140 compounds they tested for, only seven was detected in some of the larvae meal samples. Two pesticides, chlorpyrifos and piperonyl butoxide were found in one of the *M. domestica* and *C. vomitoria* samples, respectively. However, the concentrations detected were below the recommended allowance, therefore is unlikely to be a significant safety threat. A veterinary medicine, Nicarbazin, was detected in one of the *M. domestica* samples. The toxic heavy metal cadmium was detected in all the samples,

with levels exceeding the EU limit in animal feed in three of the *M. domestica* samples. Low levels of mycotoxins (Beauvericin and Enniatin) that are not believed to be a safety risk, were detected in two of *M. domestica* samples.

Therefore, when mass-rearing insects, it is important to consider the substrate selection process. To establish robust conclusions about the safety of fly larvae meal in livestock diets, it is important to improve our understanding of how variations in rearing substrates affect insect meal contamination. The concentration of contaminants in a rearing substrate and the degree of bioaccumulation in insects need to be explored. It is also important that several safety procedures be followed when mass-producing insects. Insect protein might cause feed and food safety issues if processing methods are incorrect. If thermal treatment is not implemented directly after harvesting, freezing of the product might be needed. Decontamination steps should be in place, especially if spore-forming bacteria that might survive the heating process is present (Rumpold & Schlüter, 2013a). Very little research on microbial contamination of larvae meal has been reported as is it is believed that microbial risks can be minimised by appropriate processing and handling methods (Rumpold & Schlüter, 2013a). When the microbial aspects of three samples of fresh, processed and stored whole insects were investigated, it was found that the levels of microorganisms and *Enterobacteriaceae* were high in fresh insects. Still, the numbers were significantly reduced by heat treatment. Boiling the insects for five minutes completely eliminated the occurrence of *Enterobacteriaceae*, whereas roasting them for ten minutes only reduced the numbers. It should be noted that heat treatment did not entirely inactivate microbial spores (Klunder *et al.*, 2012). Other processing techniques like high-pressure sterilisation techniques will be needed to kill the spores (Reineke *et al.*, 2012).

Insect farming can also pose environmental threats, especially if the farmed insect is considered a pest or poses a threat to plants or biodiversity; particularly if the farmed species is not endemic to the region. If the species imposes a risk, farming of it should be prohibited. If it is viewed as a pest or nuisance, correct measurements should be in place to prohibit the insect from escaping (van Huis & Oonincx, 2017).

When considering opening an insect rearing plant, the legislation of the country should be considered. Lähteenmäki-Uutela *et al.* (2017) wrote an extensive report on the legislation of several countries regarding the use of insects for feed. In South Africa, insects can be used in animal feed, but the product must be registered by the Department of Agriculture, Forestry and Fisheries DAFF and comply to the Farm Feed Act in South Africa (Act 36 of 1947). To produce and sell animal feed in the United States, registration with the U.S. food and Drug Administration (FDA) is necessary. The FDA will do an inspection to determine if the feed/insect producers comply with good manufacturing practices. All ingredients and additives intended for animal feed must be in accordance with § 348 Food Additives of the Act. In Mexico, there is no specific regulation to use insects as a feed source. Therefore the general legislation for feed will count for insects and have to comply to the following acts*: Ley Federal de Sanidad Animal*; *Reglamento de Sanidad Animal*; *Especificaciones de los alimentos para con- sumo animal", NOM-061-ZOO-1999*. In China, various insects can be used as feed additives. All new raw materials have to be added to the Feed Materials Catalogue and have to apply to hygiene and labelling standards and obtain a manufacturing licence (Lähteenmäki-Uutela *et al.*, 2017).

Using insects in animal feed in Europe is more complicated. At first, under regulation EC 999/2001 (EC, 2001) of the European Union, the use of insects as a protein source was banned for animals raised for

human consumption. This legislation prohibited all processed animal protein to be used in animal feed, except for hydrolysed protein and in some cases, fishmeal (Charlton *et al.*, 2015). An amendment was made to this legislation (EU Regulation 56/2013; EC, 2013) which allowed non-ruminant processed animal protein to be used in fish feed, but insects still did not fall under this category. Fortunately, in 2017, the European Commission approved the use of insects in fish feed (Regulation 2017/893/EC, 2017) and are currently working on authorising their use in poultry feed (Dabbou *et al.*, 2018).

2.9 Conclusion:

The use of insect meal as a sustainable, alternative protein source is becoming more globally appealing, especially in some developing countries where the cost of soya bean meal is becoming too expensive, creating problems for the economic sustainability of the poultry industry. Unfortunately, the current price of insect meal is still high. High production costs are due to manual labour, costs to optimise the processing methods and low bioconversions due to the use of sub-optimal insect substrates. For insect farms to be a profitable industry that can deliver an affordable product, automation technologies, free waste substrates and an ideal market is needed.

To create a market for insect meal in the poultry industry; a demand from feed companies, farmers and consumers is needed. To create this demand, it is important that the benefits of insect meal to the animal, farmer and consumer should be well explored, documented, and distributed among these entities. Even though extensive research has been done on the use of several insect species as an alternative protein source in the diets of different animal species, relatively little is known regarding its possible secondary beneficial effects (such as health promotion) in poultry, as most of the immunological and antimicrobial research has been focussed on fish species. To the best of our knowledge, research on the use of CC larvae meal in poultry diets is limited, and no published data exist on its immunomodulatory effects and possible *in vitro* antimicrobial properties. Therefore, further research is warranted on the immunomodulatory and *in vitro* antimicrobial properties of both BSF and CC larvae meals when used as a protein source in poultry diets.

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Chapter 3

Influence of substrate on the nutrient composition of *Hermetia illucens* and *Chrysomya chloropyga* larvae meal, and its acceptability to broilers

Abstract

Insects have been proposed as a sustainable, high-quality protein source. Major focus has been placed on *Hermetia illucens* (BSF) larvae on the breakdown of organic matter from waste streams to produce high-quality protein. However, research on carrions which are excellent in converting animal offal, are scarce. The nutritional composition of *Chrysomya chloropyga* larvae meal (CCm) reared on animal offal, and BSF meal reared on three different substrates: a variety of kitchen waste (BSF-CWm); 100% commercial layer chicken mash (BSF-Mm); or 50% commercial layer chicken mash + 50% fish offal (BSF-F)) were investigated. The acceptability of the BSF-CWm and CCm meal by broiler chickens was determined by a feed choice trial. *Chrysomya chloropyga* larvae meal had a high crude protein content (57%) with a moderate fat content (23.7%). Rearing substrate had a minimal effect on the protein content of BSF larvae, as the crude protein of the three BSF sources ranged from 33.9% to 34.3%. The amino acid composition was affected by Diptera species and feeding substrate. *Chrysomya chloropyga* had the highest lysine (4.18%) and methionine (1.39%) content. Between BSF larvae meal sources, BSF-CWm had the highest methionine content (0.69%), while BSF-Fm possessed the highest lysine content (2.24%). When considering the essential amino acid index (EAAI), CCm provided essential amino acid levels closest to the requirements of modern broilers, whereas an oversupply of essential amino acids was most prominent in BSF-Fm meal. Methionine was the first limiting essential amino acid in all three sources of BSF meal while threonine was first in CCm meal. For the BSF sources, most of the EEA's (except for methionine and leucine) compared well to the ideal amino acid profile of broilers, whereas CCm was slightly deficient in all the EEA's (except for histidine, phenylalanine, and tyrosine), but this is mostly due to its high lysine content (4.18%). Even though broilers preferred diets containing 10% BSF or 10% CC meal over maize-soya-based diets, an overall preference was observed for diets containing CCm meal. Based on the nutrient profile of the larvae meal sources and its acceptability by broilers, CC meal, as well as all the BSF meals, have the potential to be used is as an alternative protein source in broiler diets.

Keywords: Essential amino acid index, ideal amino acid ratio, protein quality, feed choice

3.1 Introduction

Plant and animal protein sources are the two most important protein sources used in poultry diets. It is vital that protein sources used in monogastric animal diets have a high crude protein content with an adequate amino acid profile. It should also be palatable, highly digestible and free of anti-nutritional factors (Sánchez-Muros *et al.*, 2014). Soya bean meal is the most widely used plant protein source in poultry diets because of its high protein content and well balanced essential amino acid profile, enabling it to balance most cereal-based diets (Ravindran, 2013). In general, plant proteins are unbalanced and have deficiencies in certain essential amino acids, decreasing their nutritional value because they might not supply the animal with the essential amino acids required for optimal growth and production (Beski *et al.*, 2015). On the other hand, animal proteins are usually balanced in terms of essential amino acids but are expensive. In broiler diets, especially starter diets for young chicks with high protein and essential amino acid requirements, a fraction of plant protein are more often than not substituted with an animal protein source or pure amino acids to create a balanced diet (Ravindran, 2013). The banishment of certain animal protein sources (due to diseases such as bovine spongiform encephalopathy), together with the decrease in marine fish and increase in fishmeal prices, heightens the need for alternative, sustainable, high-quality protein substitutes.

Insects have been proposed as a sustainable, high-quality protein source (Bosch *et al.*, 2014). There are approximately one million species of insects, and the nutrient composition of only a small fraction of the species has been determined (Sánchez-Muros *et al.*, 2014). Various insect species have been explored for their use in feed and food, with the most popular species for animal feed being house fly (*Musca domestica*) larvae, BSF larvae (BSF larvae), crickets, grasshoppers and mealworms (EFSA Scientific Committee, 2015). The ability of Diptera species' larvae to turn low-grade bio-waste into high-quality protein sources has made them a popular topic to explore in recent years. Different insect species have diverse feeding habits and can be fed on several waste streams such as restaurant surpluses, manure, cereal remnants, or offal from slaughterhouses (Nguyen *et al.*, 2015; Parry, 2017). Protein content and amino acid composition of insects can vary greatly due to the species, time of harvest (life stages) (Veldkamp & Bosch, 2015) and the substrate used to rear the insects on (Nguyen *et al.*, 2015). Major research foci have been on BSF larvae and its ability to break down matter from organic waste streams to produce high-quality protein. However, waste conversion is severely affected when BSF larvae are reared on animal offal alone (Nguyen *et al.*, 2015). Therefore, there is a need to explore carrion Diptera species for offal waste recycling of which the larvae meal can ultimately be used as a protein source in animal feed. The larvae of *Chrysomya Chloropyga* (CC), a blow fly native to Africa, has a higher protein content and essential amino acid concentration compared to BSF larvae meal (Haasbroek, 2016). *Chrysomya chloropyga* larvae are carrion feeders, making them excellent in converting animal offal (Parry, 2017), but published data on its use in monogastric animals is scarce. Therefore, this study aims to describe the nutrition value of CC larvae, as well as BSF larvae (reared on three different substrates including animal waste) and to determine the acceptability of these two species when included in broiler diets.

3.2 Materials and methods

3.2.1 Insect farming

Chrysomya chloropyga (CC) flies were caught on Mariendahl experimental farm, Stellenbosch, to start a colony. To trap the flies, the bottom of a red-top flycatcher was cut out, and a 500ml container covered with mesh was attached to the base with an elastic band. Chicken livers were placed in the container to serve as bait. The bait was used for four consecutive days, but flies were collected every 24 hours. The holder with bait was removed to separate CC flies from the other species caught in the trap. The trap was placed in a refrigerator at 4°C for two minutes to immobilize the flies temporarily for subsequent selection of CC flies.

The selected CC flies were placed in 2m x 1m x1m netted cages in an environmentally controlled room (temperature of 27 ± 3°C and relative humidity of 65 ± 5%). Each cage was supplied with a poultry bell drinker for water. A cloth was placed in the bottom of the drinker to absorb excess water and prevent the flies from drowning. Flies were supplied with dry calf milk powder (Volac milk replacer) and sugar to serve as a protein and energy source. For oviposition to take place, flies were supplied with swine offal. Eggs were removed every eight hours. Vertical stacking trays (60 x 40 x 20cm) equipped with metal rods were used to rear the larvae in. Six stacking trays were placed in a plastic container (100 x 100 x 40cm). The plastic container was used for the collection of migrating larvae. The inner sides of the collection container were lightly dusted with cornflour to prevent the migrating larvae from escaping.

Eggs were placed on swine offal consisting out of liver, lungs, kidneys, hearts, and spleens. The substrate was sprayed with water twice daily to prevent it from drying out. Larvae were reared on the substrate for about five days and collected from the collection tray after migration from the substrate took place. Larvae (250g) were killed by being placed in 2L boiling water for one minute. Larvae were rinsed three times, dried in a commercial ventilated drying oven until dry matter content reached 92% (± eight hours) where after the larvae were minced into a fine meal (± 2mm) in a commercial food processor.

Three sources of black soldier fly larvae meal, namely, BSF-CWm, BSF-Fm, BSF-Mm were used for this study. The BSF-CWm larvae meal was commercially produced by Agriprotein (Pty) Ltd (Cape Town, South Africa) on an unspecified variety of kitchen waste. Larvae were harvested before the pre-pupae stage (16 days) were reached. Harvested larvae were dried on a fluidized bed dryer, milled, and packaged. Freshly hatched larvae used to produce the BSF-Mm and BSF-Fm larvae meal were obtained from Agriprotein (Pty) Ltd (Cape Town, South Africa). To produce the BSF-Mm larvae meal, BSF neonatal larvae were reared on a commercial chicken feed (CP = 130g/kg; fat = 25 g/kg; fibre = 70 g/kg; moisture = 120 g/kg; Ca = 35 g/kg; P = 5 g/kg; lysine 5 g/kg), previously soaked in hot water (water:mash = 1.6:1) for 16 hours. For the BSF-Fm larvae meal, a substrate consisting out of 50% soaked chicken feed + 50% fish offal were fed to the larvae. The moisture content of the substrate was monitored daily, and if needed, water was added to maintain the ideal moisture content of 60%. Larvae were reared in an environmentally controlled room (temperature of 27 ± 1°C and relative humidity of 65 ± 5%) and harvested after 16 days, before reaching 6th instar (prepupae). Larvae were separated from the remaining residue by emptying the containers into a saturated saltwater solution, in which the larvae stayed afloat. Larvae were placed in boiling water for one minute to kill them, inhibit tyrosinase activation, and autolysis. Subsequently, larvae were rinsed to remove all the salt and debris, dried in a

ventilated drying oven for 16 hours at 65°C until dry matter content reached 92%. Dried larvae were minced into a fine meal (± 2 mm) using a commercial food processor.

3.2.2 Proximate analysis of larvae meal

3.2.2.1 Dry matter content

The dry matter (DM) content of the larvae meal sources was determined according to the AOAC method 934.01 (AOAC 2002). Two replicate samples weighing 2 g each were placed in a pre-dried crucible of which the dry weight was determined. Samples were dried at 100°C for 24 hours. Subsequently, samples were placed in a desiccator for 30 minutes, weighed on a Mettler AE 200 scale (Mettler-Toledo, Switzerland) with 0.0001 g accuracy.

3.2.2.2 Ash content

The ash content was determined according to Official AOAC Method 942.05 (AOAC, 2002). The samples retained after DM determination were kept in the crucibles and placed in a furnace for six hours at 500°C.

3.2.2.3 Crude protein (CP) content

The crude protein (CP) content of the larvae meal sources was determined by measuring the total nitrogen (N) content according to the Official AOAC Method 4.2.07 (AOAC, 2002) using a LECO FP528 apparatus. Duplicate samples (0.1 g) were weighed in a tin cup and placed into the LECO analyzer. After that, the N content taken from the LECO FP528 was multiplied by a factor of 6.25 to obtain crude protein percentage. Amino acid content was determined at the Food and Feed laboratory (SANAS accredited) of the Agricultural Research Council ARC-Irene analytical services. Amino acid content was determined utilizing acid hydrolysis extraction, followed by pre-column derivitization, and separation by High-Performance Liquid Chromatography (HPLC).

3.2.2.4 Crude fat content

Crude fat was determined through acid hydrolysis, method 954.02 (AOAC 2002). Duplicate samples with a weight of 2 g each were added to a test tube, followed with 2 ml of ethanol and 10 ml HCL solution (3%). Test tubes were boiled for 30 minutes in a water bath after which the mixture was poured into a separating funnel. The tube was rinsed with 10ml ethanol and subsequently adding it to the funnel. A volume of 25 mL of diethyl ether was added to the funnel and shaken for one minute. Subsequently, 25 mL of petroleum ether was added, and the mixture was shaken for one minute. The transparent upper portion of liquid was transferred into a fat beaker. This shaking process was repeated twice more. Fat beakers were placed in a warmed sand bath until all the ether evaporated. Beakers were weighed to determine fat content.

3.2.2.5 Crude fibre content

Glass crucibles containing 1 g of sample (in duplicate) was placed into a Fibertec/Dosifiber extrusion apparatus. Acetone was used to de-fat the samples. Samples were boiled in 0.128 M sulphuric acid, followed by boiling in 0.313 M sodium hydroxide. Samples were dried at 100°C for 24 hours, weighed, placed in a furnace for 6 hours at 500°C and subsequently weighed again.

3.2.3 Protein scores of larvae meal

During this study, the nutritional value of the larvae meal proteins was evaluated by: 1) calculation of the essential amino acid index (EAAI), 2) calculation of the chemical scores of each source, 3) expressing amino acid profiles as a percentage of lysine. For EAAI and chemical score calculations, the percentage amino acids in each source were converted to mg per g crude protein (aa) (Table 3.3). The amino acid requirements for the animal were also converted to mg/g CP requirement (AA). The chemical scores for each essential amino acid was calculated by taking the ratio between the amino acid content of the protein source (mg/g CP) and the requirement of the intended animal (mg/g CP required) as demonstrated in equation 3.1 (Rama Rao *et al.*, 1960; Veldkamp & Bosch, 2015). In other terms, each amino acid in the protein source (in % of crude protein) was divided by this amino acid requirement of the target animal (in % of crude protein) and multiplied by 100 to determine the chemical score for each amino acid. The EAAI is a rapid method to evaluate the amino acid composition of a raw material. It considers the concentration of each essential amino acid (mg/g CP) in the protein source and the requirement for these amino acids in the animal (mg/g CP required) (Table 3.3). An EAAI value of one is an indication that the amino acid profile, in general, meets the requirement of the target animal. A value greater than one means there is a protein overload while values less than one is an indication that the amino acids are far lower than the protein requirement of the animal. The EAAI of each source was determined using equation 3.2

Equation 3.1:

$$CS = \frac{aa1}{AA1}$$

Where:

$$aa = \frac{amino\ acid\ in\ source\ (g\ per\ 100g) \times 1000}{CP\ content\ of\ source\ (\ g\ per\ 100g)} = mg\ amino\ acid\ per\ gram\ CP$$

$$AA = \frac{amino\ acid\ requirement\ (g\ per\ 100g) \times 1000}{Crude\ protein\ requirement\ (g\ per\ 100g)} = mg\ amino\ acids\ required\ per\ gram\ protein$$

Equation 3.2:

$$EAAI\ = \sqrt[n]{\left(\frac{aa1}{AA1} \times \frac{aa2}{AA2} \times \frac{aa3}{AA3} \times \frac{aa4}{AA4} \times \ldots \frac{aan}{AAn}\right)}$$

Where:
n = number of essential amino acids used in calculation

3.2.4 Feed choice trial

A two-way feed choice (preference) trial was conducted at Mariendahl trial unit, Stellenbosch, to determine the acceptability of CCm and BSF-CWm meal. A total of 24 one day old ROSS 308 broilers were placed in one cage for an adaption period of ten days. Birds had *ad-lib* access to all three treatment diets (Table 3.1). To obtain three dietary treatments (CON; BSF-CW and CC); iso-nitrogenous and iso-caloric diets were formulated by partly substituting soya bean meal and full-fat soya in the maize-soya-based control diet (CON) with 10% BSF-CWm larvae meal (BSF-CW) or 10% CCm larvae meal (CC). After the ten days adaption period, chicks were randomly allocated to 24 single pens (one bird per cage). Eight cages were provided with two feeders containing the CON diet and BSF-CW diet to determine if chicks prefer larvae meal diets over the control diet. Another eight pens were provided with the CON and CC diet. To determine if chickens will prefer one of the larvae meal sources, the remaining eight cages were each provided with two feeders containing the BSF-CW and CC diets. Each feeder was refilled three times daily to prevent feeders from becoming empty. A small tray was placed under each feeder for the collection of any spilled feed. Feeders were placed at complete randomized order in the cage, and their position was changed daily. Feed intake per cage for each diet was recorded after ten days. The intake of each diet per bird was expressed as a percentage of the total intake per bird.

3.2.5 Statistical analysis

Preference values were compared by ANOVA using the GLM procedure. Means from the ANOVA models were compared using Fishers least significant difference test.

Table 3.1 Ingredient and calculated nutrient composition of the feed choice trial diets

Ingredients	Units	CON[1]	CC[2]	BSF[3]
Maize	%	50.64	56.71	52.35
Soya bean meal (Full fat)	%	26.00	13.30	11.00
Soya bean meal (46% CP)	%	17.84	15.41	22.50
L-lysine (HCl)	%	0.266	1.191	0.306
DL-methionine	%	0.382	0.251	0.336
L-threonine	%	0.120	0.102	0.098
Premix	%	0.250	0.250	0.250
Limestone	%	1.399	1.626	1.017
Salt	%	0.266	0.092	0.236
Mono-calcium phosphate	%	1.981	1.767	1.617
Sodium bicarbonate	%	0.133	0.304	0.288
Sunflower oil	%	0.727	0.000	0.000
Hermetia illucens larvae meal (BSF-CWm)	%	0.000	0.000	10.00
Chrysomya chloropyga larvae meal (CCm)	%	0.000	10.00	0.000
Calculated nutrient composition				
Dry matter	%	88.64	88.70	88.73
AMEn chick[4]	MJ/kg	12.55	12.55	12.55
Crude protein	%	22.80	23.00	22.80
Crude fibre	%	3.436	3.487	3.815
Crude fat	%	7.721	7.146	8.193
Lysine	%	1.472	1.473	1.463
Methionine	%	0.711	0.652	0.690
Cysteine	%	0.384	0.423	0.465
Methionine + Cysteine	%	1.095	1.075	1.155
Threonine	%	0.986	0.988	0.984
Tryptophan	%	0.266	0.218	0.282
Arginine	%	1.536	1.555	1.549
Isoleucine	%	1.022	0.990	1.019
Leucine	%	1.952	1.929	1.945
Histidine	%	0.614	0.638	0.613
Phenylalanine	%	1.048	1.104	1.026
Tyrosine	%	0.845	0.834	0.863
Valine	%	1.127	1.159	1.184
Ash	%	4.394	4.355	4.324
Calcium	%	0.960	1.000	1.000
Total phosphorous	%	0.904	0.852	0.843
Available phosphorous	%	0.480	0.500	0.480
Sodium	%	0.160	0.230	0.198
Chloride	%	0.250	0.245	0.250
Potassium	%	0.950	0.795	0.878

[1] CON = control diet; [2]CC = diet containing *C. chloropyga* larvae meal; [3]BSF = diet containing *H. illucens* larvae meal
[4]AMEn = Nitrogen-corrected apparent metabolisable energy

3.3 Results and discussion

3.3.1 Chemical composition and protein quality analysis

Larvae species had a pronounced effect on the nutrient composition (Table 3.2) of larvae meal. *Chrysomya chloropyga* (CC) larvae consisted out of ± 34 g/100g more crude protein and ± 14 g/100g lower crude fat content compared to the three BSF larvae meal sources. The rearing substrate in the current study had a minor effect on crude protein (32.9-34.3%) and crude fat (34.2-38.3%) content of BSF larvae meal. In contrast, Barragan-Fonseca *et al.* (2017) reported crude protein levels for BSF to vary from 37% to 63% (DM basis), depending on their substrate. It should be noted that the studies included in their review did not specify the age of harvesting, and the authors used different analytic methods to determine crude protein, which might be attributable to differences between studies. Interestingly, authors (Driemeyer, 2016; Haasbroek, 2016; Cockcroft, 2018) whom used different kitchen waste substrates, but harvested at a similar age and used the same analytic methods to determine crude protein as in this study, reported comparable crude protein values (35.0%, 35.9%, and 36.6%, respectively). Furthermore, Haasbroek, (2016) reported similar crude protein values for CC larvae meal (58.6 vs 57.0% in this study). Even though the fat content between BSF larvae only varied slightly, Driemeyer (2016) and Haasbroek (2016) who used similar analytic methods to determine fat content, reported crude fat levels of 48.1%, 40.1%, respectively. Therefore these results (Table 3.2) are in agreement with Barragan-Fonseca *et al.*, (2017), who concluded that substrate has a prominent effect on the fat content of BSF larvae. This phenomenon might be explained by the fact that BSF flies have a non-feeding adult stage, the larvae on high-fat diets accumulate as much fat as necessary for complete development (Nguyen *et al.*, 2015). Even though the substrate had minimal effect on the protein content of BSF in the current study, a greater effect was observed on the amino acid content of the three different BSF larvae meals (Table 3.2). *Chrysomya chloropyga* meal (CCm) is a good source of methionine and lysine, which are usually considered to be the first and second limiting amino acid in conventional maize-soya-based broiler diets.

Several methods exist to evaluate the nutritional value of the protein in raw materials. During this study, the nutritional value of the larvae meal proteins was evaluated by calculation of the essential amino acid index (EAAI); calculation of the chemical scores of each source; and expressing amino acid profiles as a percentage of lysine. The EAAI is the adequacy between the concentration of all the essential amino acids in the dietary protein and the requirement of the target animal. When considering the protein and essential amino acid requirements for Ross 308 broiler chickens as supplied by Aviagen, (2014), the EAAI values (Table 3.3) for all the protein sources used in this study were all greater than one. This value is an indication that all the studied protein sources provide more essential amino acids than required by broilers. The EAAI for CCm meal was the closest to one, followed by FM and then SBM. The EAAI index for BSF larvae reared on fish offal (BSF-Fm) were the highest, followed with BSF-CWm and BSF-Mm. These results indicate that CCm, FM and SBM provide levels of essential amino acids closest to the requirements of broiler chickens. Previously reported EAAI's for BSF larvae ranged between 1.43 and 1.50 (Veldkamp & Bosch, 2015; Huang *et al.*, 2018).

Table 3.2 Nutritional composition (dry matter basis) of *Chrysomya chloropyga* larvae meal (CCm) and *Hermetia illucens* larvae meal (BSF-CWm, BSF-Fm and BSF-Mm) reared on different substrates

	CCm[1]	BSF-CWm[2]	BSF-Mm[3]	BSF-Fm[4]
Proximate analysis (%)				
Dry matter (As is)	93.67	92.60	93.40	94.01
Crude protein	60.85	35.53	35.97	36.49
Crude fat	25.30	41.36	36.62	39.04
Fibre	7.87	10.08	5.93	5.46
Ash	5.25	12.85	14.45	10.55
Amino acid composition (g/100g larvae meal)				
Lysine	4.46	1.93	2.01	2.38
Methionine + Cysteine	2.58	2.13	3.32	1.91
Methionine	1.48	0.75	0.54	0.67
Tryptophan	0.26	0.63	0.67	0.73
Arginine	4.58	2.81	2.04	4.03
Threonine	2.50	1.64	1.42	1.76
Valine	3.15	2.32	1.85	2.26
Isoleucine	2.51	1.68	1.40	1.63
Leucine	3.83	2.58	2.18	2.32
Histidine	2.26	0.96	1.43	2.26
Phenylalanine +Tyrosine	6.10	3.54	3.62	4.49

[1]CCm = meal from *C. chloropyga* larvae reared on animal offal; [2]BSF-CWm = meal from BSF larvae reared on kitchen waste; [3]BSF-Mm = meal from BSF larvae reared in chicken feed; [4]BSF-Fm: meal from BSF larvae reared on 50% chicken feed + 50% Fish offal

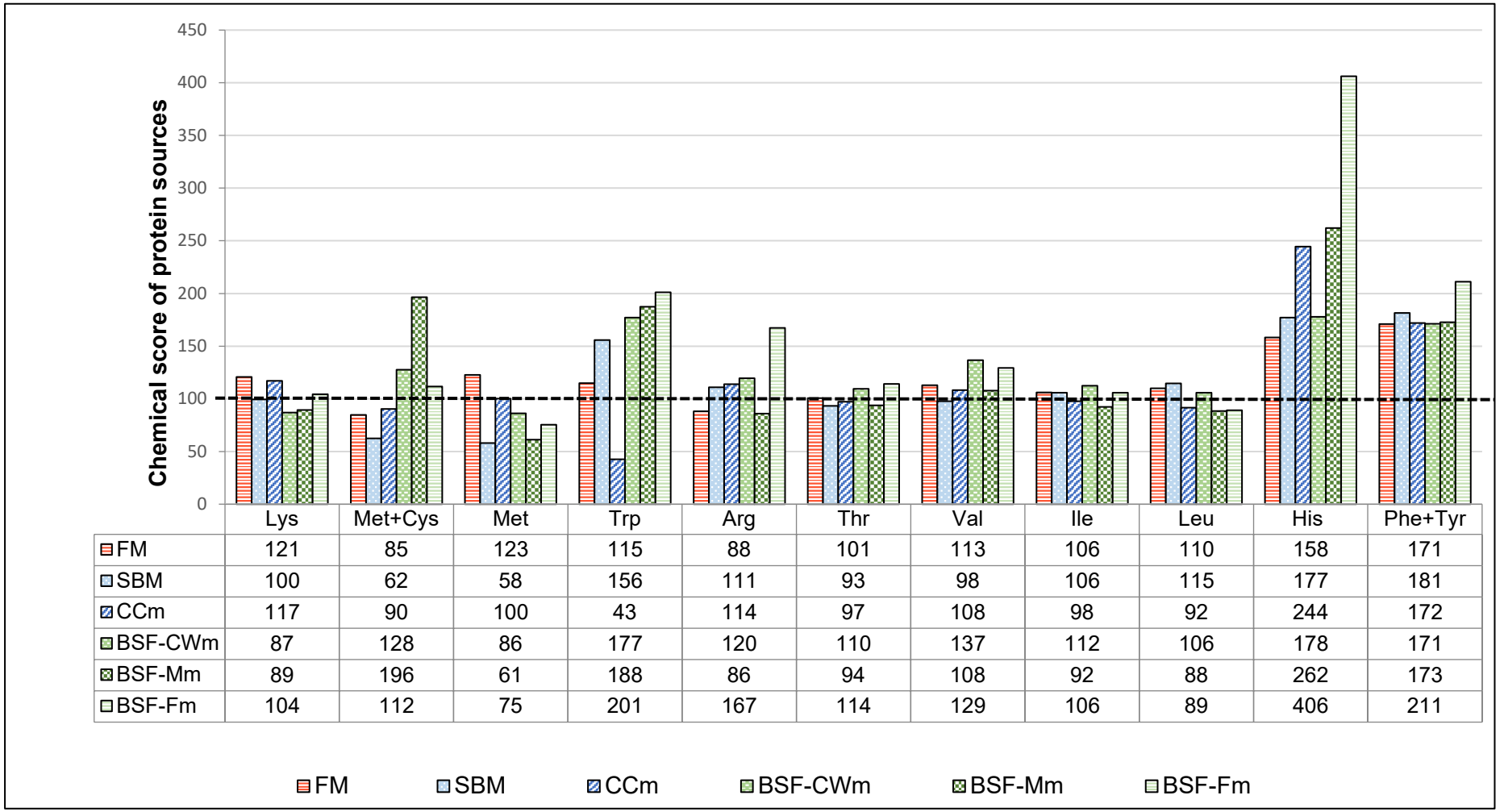
When a diet is inadequate in any essential amino acid, protein synthesis cannot proceed beyond the level at which that essential amino acid is available. That amino acid is thus the limiting amino acid for the protein source. Also, the amino acid with the lowest chemical score in a protein source is the limiting amino acid. The chemical scores for FM, SBM, CC, BSF-CWm, BSF-Mm and BSF-Fm were 85, 58, 43, 86, 61 and 75, respectively (Figure 3.1). Methionine was the first limiting amino acid (lowest chemical score) for SBM and the three BSF sources. As a result, supplementation of pure methionine will be inevitable when the studied BSF larvae meal sources are used in maize-soya-based diets intended for modern broilers with a 23% crude protein requirement. In this study, a mixture of kitchen waste as a substrate for BSF larvae resulted in the highest level of methionine between the three BSF sources.

Table 3.3 Amino acid requirement of modern broilers, amino acid composition of larvae meal sources (expressed as milligram per gram crude protein) and the essential amino acid index (EAAI)

	Ross 308[1] (requirements)	FM	SBM	CCm	BSF-CWm	BSF-Mm	BSF-Fm
Amino acid composition (mg/g CP)							
Lysine	62.6	75.7	62.3	73.3	54.4	56.0	65.3
Methionine + Cysteine	47.0	39.8	29.3	42.5	59.9	92.3	52.5
Methionine	24.3	29.9	14.1	24.4	21.0	14.9	18.4
Tryptophan	10.0	11.5	15.6	4.3	17.7	18.8	20.1
Arginine	66.1	58.2	73.3	75.3	79.0	56.8	110.5
Threonine	42.2	42.5	39.4	41.1	46.2	39.6	48.1
Valine	47.8	53.9	46.7	51.8	65.3	51.5	61.8
Isoleucine	42.2	44.7	44.6	41.2	47.4	39.0	44.6
Leucine	68.7	75.5	78.7	63.0	72.6	60.7	61.2
Histidine	15.2	24.1	26.9	37.2	27.1	39.9	61.8
Phenylalanine +Tyrosine	58.3	99.6	105.7	100.2	99.7	100.6	123.0
Essential amino acid index (EAAI)							
	NA	1.13	1.14	1.07	1.29	1.27	1.47

[1] Requirements for 308 broilers as stated in Aviagen (2014); FM = fishmeal (NRC, 1994), SBM = soya bean meal (NRC, 1994), CCm = meal from *C. chloropyga* larvae reared on animal offal; BSF-CWm = meal from BSF larvae reared on kitchen waste; BSF-Mm = meal from BSF larvae reared in chicken feed; BSF-Fm: meal from BSF larvae reared on 50% chicken feed + 50% fish offal

The sole use of commercial chicken feed as a substrate for the larvae lessened the methionine levels of the BSF-Mm meal. Adding 50% fish offal to the layer mash or using a variety of kitchen waste increased the methionine concentration slightly in BSF-Fm, but this amino acid was still limited. Similarly, Veldkamp & Bosch (2015), who used BSF composition data available from electronic literature searches, reported a chemical score of 68 for BSF, with methionine as the first limiting amino acid. The lowest chemical score for all the protein sources in the current study was for CCm larvae meal (Figure 3.1). Interestingly, unlike the BSF sources, tryptophan was the first limiting amino acid in CCm larvae meal, even though the chemical score for methionine was 100. The fact that CC are deficient in tryptophan and contains higher levels of methionine and lysine makes it ideal to use as a protein supplement in maize-soya-based diets, which are usually deficient in methionine and lysine but have sufficient tryptophan levels



	Lys	Met+Cys	Met	Trp	Arg	Thr	Val	Ile	Leu	His	Phe+Tyr
FM	121	85	123	115	88	101	113	106	110	158	171
SBM	100	62	58	156	111	93	98	106	115	177	181
CCm	117	90	100	43	114	97	108	98	92	244	172
BSF-CWm	87	128	86	177	120	110	137	112	106	178	171
BSF-Mm	89	196	61	188	86	94	108	92	88	262	173
BSF-Fm	104	112	75	201	167	114	129	106	89	406	211

Figure 3.1 Calculated chemical score for larvae meal in modern Ross 308 broilers (Aviagen, 2014). Studied sources were: FM = fishmeal (NRC, 1994), SBM = soya bean (NRC, 1994), CCm = meal from *C. chloropyga* larvae reared on animal offal; BSF-CWm = meal from BSF larvae reared on kitchen waste; BSF-Mm = meal from BSF larvae reared in chicken feed; BSF-Fm: meal from BSF larvae reared on 50% chicken feed + 50% fish offal

Dietary requirements of broilers are essential for the calculation of chemical score and EAAI. Provided that dietary, environmental and genetic factors may affect amino acid requirements, essential amino acids can be expressed as an ideal ratio to lysine, since the ratio remains mostly unaffected by these variables (Emmert & Baker, 1997). When considering the amino acid profile expressed as a ratio of lysine (Figure 3.2), the ratio with methionine in FM, followed by BSF-CWm and CCm, resembles the requirements of broilers the closest, with methionine/lysine being the lowest for SBM. Similar ratios for sulphur-containing amino acids (SAA = methionine + cysteine) were observed for FM, SBC and CCm meal, but cysteine levels in the BSF meal sources were high, resulting in very high SAA/Lys ratios. Even though cysteine can be synthesized through the trans-sulphuration pathway from methionine, methionine cannot be synthesized from cysteine (Pillay *et al.*, 2006). The high levels of cysteine will therefore not compensate for the deficiency of methionine in the SBM and the BSF sources, but the high dietary cysteine contribution from BSF may lower the need for synthetic methionine when included in cysteine deficient diets, such as maize-soya-based diets. The Arginine/lysine ratio for SBM, CCm, and BSF-Mm are similar and closely fits the ratio required for poultry feeds, but ratios in BSF-CWm and BSF-Fm were in excess. The ratios lysine to threonine, valine and isoleucine are similar in all the protein sources and compare well with the ratio for poultry feed requirements. However, the ratios for BSF-Mm are the highest and slightly higher than required. Histidine and phenylalanine + tyrosine are abundant in all the protein sources investigated, with the highest levels in the BSF larvae meal sources. Their abundance is also reflected in the high EAAI for BSF sources. It should be noted that an overload in amino acids is not always desired due to energy requirements for amino acid deamination and uric acid production, an excess or imbalance of amino acids will reduce the dietary energy available for production, which can ultimately have a negative effect on growth and production (Wu, 2017)

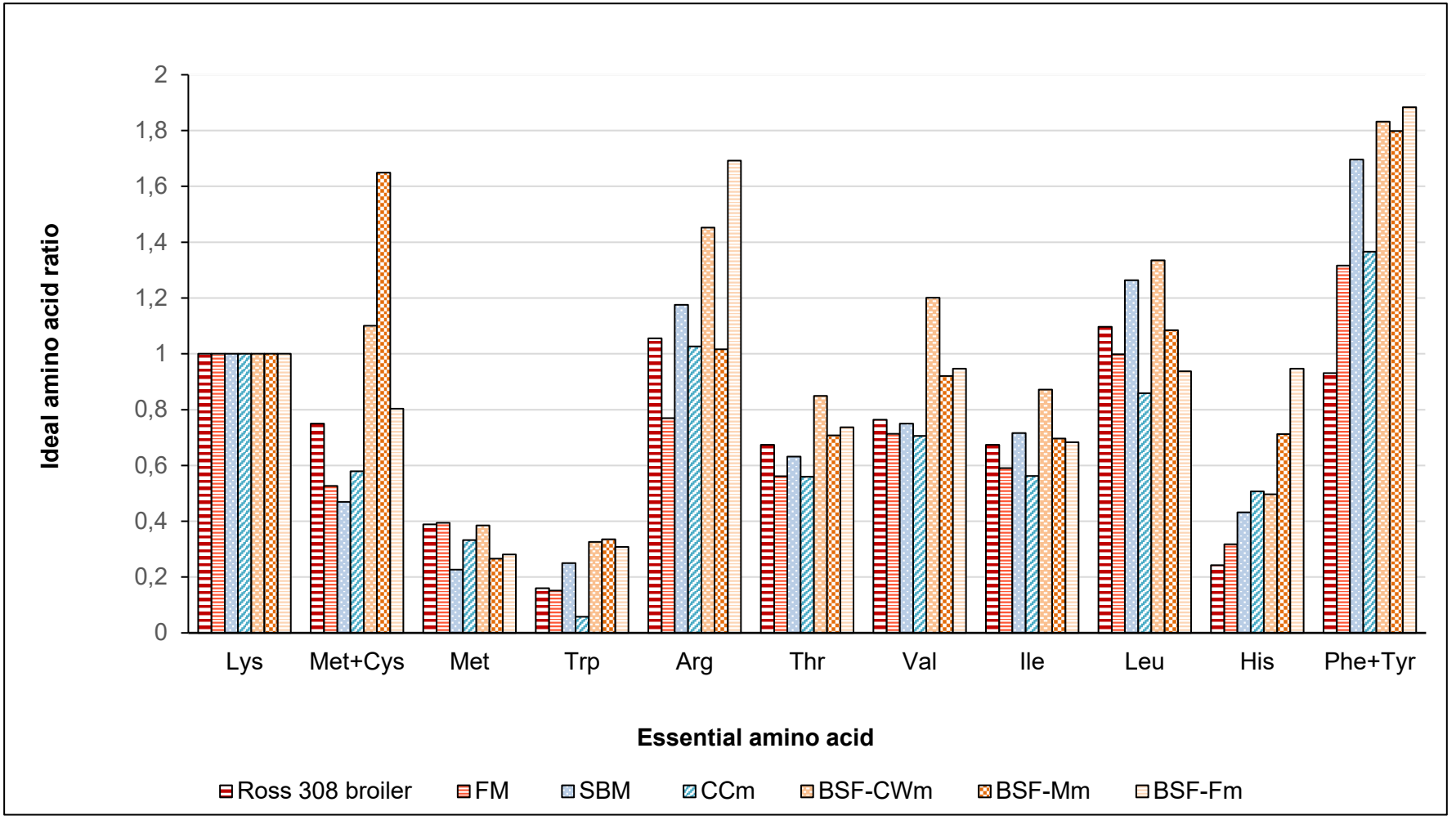


Figure 3.2 Calculated amino acid to lysine ratios of the studies protein sources in comparison to the ideal amino acid profile for modern Ross 308 broilers (Aviagen, 2014). Studied sources were: FM = fishmeal (NRC, 1994), SMB = soya bean meal (NRC, 1994), CCm = meal from *C. chloropyga* larvae reared on animal offal; BSF-CWm = meal from BSF larvae reared on kitchen waste; BSF-Mm = meal from BSF larvae reared in chicken feed; BSF-Fm: meal from BSF larvae reared on 50% chicken feed + 50% Fish offal

3.3.2 Feed choice trial

When evaluating the feed type available (Table 3.1) and their choice for consumption, the chickens when given a choice between the CON diet or diets containing CC larvae meal (Figure 3.3a), preferred CC larvae meal (P = 0.0001). Broilers also preferred the BSF treatment over the CON diet (P = 0.0014) (Figure 3.3b). Interestingly, when broilers had a choice between either of the larvae meal diets, a preference was observed towards the CC diets (P < 0.0001). Poultry eat to satisfy their energy requirements when they receive a balanced diet (Nutrient requirements of poultry, 1984). However, in a free-choice situation, chickens prefer certain raw materials over others, and the feeding preference does not necessarily correlate with the nutritional value of the raw materials (Kare & Scott, 1962). When chickens receive similar iso-nitrogenous and iso-caloric diets, they preferred diets containing feedstuffs with unidentified growth factors (Alenier & Combs, 1981). Therefore, aside from the known nutritional value, the preference by chickens for one raw material over the other can be due to unidentified growth factors and by palatability, which is the integrated response to taste, aroma, texture, colour or shape of raw materials (Alenier & Combs, 1981).

As maize-soya-based diets are known to be palatable for poultry, preference for diets containing BSF-CW and CC larvae meal could demonstrate a preference for larvae meal rather than avoidance of the control diet. In this study, broilers preferred the CC diets over the BSF diets (65% vs 35%) (Figure 3.3c). The CC and BSF diets had the same nutritional value and ingredients (except for the different larvae species). The texture, colour and shape of the grounded larvae were also similar. Therefore, preference for the CC diet is more likely due to aroma, taste or unidentified growth factors in the CC larvae meal. Even though there are currently no feed preference studies on diets containing *C. chloropyga* larvae meal, Cullere *et al.*, (2016) also observed a preference towards diets containing BSF larvae meal rather than a commercial maize-soya-based diet during a feed choice trial on broiler quails.

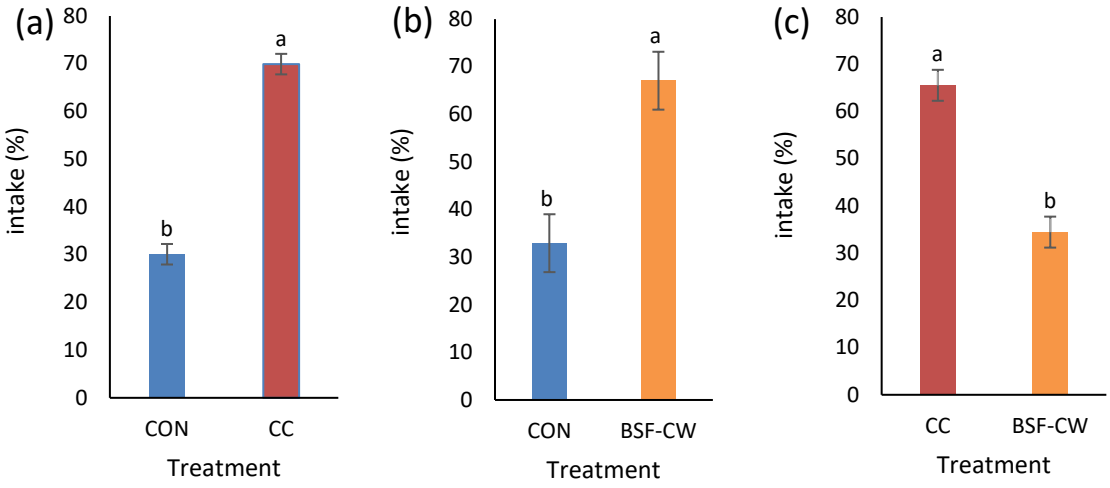


Figure 3.3 Diet preference of broilers receiving two treatment diets per cage for ten days: a) broilers receiving the control diet (Con) and a diet containing 10% *C. chloropyga* larvae meal (CC); b) broilers receiving the control diet (Con) and a diet containing 10% *H. illucens* meal (BSF-CW); c) broilers receiving CC diet and BSF-CW diet [a,b] Means with different superscripts within each graph differ significantly (P <0.05)

3.4 Conclusion

Between all of the larvae meal sources, CCm meal contained the highest crude protein and lowest fat content. In this study, BSF larvae maintained a form of protein homeostasis, regardless of its rearing substrate. The amino acid composition was affected by the Diptera species and the feeding substrate of BSF larvae. *Chrysomya chloropyga* meal had the highest lysine and methionine content, making it ideal for including in conventional maize-soya-based broiler diets which are usually considered limiting in these two amino acids. Even though broilers preferred diets containing 10% BSF or 10% CC meal over maize-soya-based diets, an overall preference was observed for diets containing CC meal. Based on the nutrient profile of the larvae meal sources and its acceptability by broilers, CC meal as well as all the BSF meals have the potential to be used as an alternative protein source in broiler diets. Further studies are needed to determine the effect of CC larvae meal on production parameters and health of broiler chickens.

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Chapter 4

Effect of dietary *Chrysomya chloropyga* and *Hermetia illucens* larvae meal on the growth performance, haematological parameters, humoral and cell-mediated immune response of broiler chickens

Abstract

Various insect species have been explored for their use in feed and food, with *Hermetia illucens* (BSF) larvae being one of the most popular species studied. Research is scarce on carrion Diptera species, such as *Chrysomya chloropyga* (CC), who are excellent in converting animal offal. This study assessed the safety and immunomodulatory properties from the use of BSF and CC larvae meal in broiler diets. A total of 108 broilers chicks were randomly assigned to six treatment groups given either a control diet (CON), a control diet supplemented with antimicrobial growth promotor, Zinc Bacitracin (ZincBac), a diet containing 10% or 15% CC larvae meal (10%CC and 15%CC, respectively), a diet containing 10% or 15% BSF larvae meal (10%BSF and 15%BSF, respectively). Each treatment group consisted of 18 replicate cages with one bird per cage. To determine the humoral immune response, broilers were injected twice with sheep red blood cells (SRBC), and serum antibody titres were determined to assess the degree of the primary and secondary immune responses. As a measure of cell-mediated immunity, the thickness of the toe web was measured 24 hours after injection of phytohemagglutinin-P (PHA-P). On 35 days of age, no differences in body weight or feeding intake were observed between treatment groups, but birds receiving 15% BSF had a poorer feed conversion ratio (FCR) than birds in the CON group, though differences diminished on day 42. At slaughter on day 42, organs were collected and weighed, pH of the gastrointestinal tract and liver colour was measured. Dietary larvae meal had no adverse effects on haematological parameters, lymphoid organ weights, liver colour or gastrointestinal pH. The 10% inclusion of BSF or CC meal significantly increased the primary humoral immune response and cell-mediated immunity. Based on production parameters, haematological parameters, and lymphoid organ weights, BSF and CC larvae meal was non-toxic, safe to use as an alternative protein source and holds health benefits to the broilers at a 10% dietary inclusion level.

4.1 Introduction

Due to the emergence of bacterial resistance to antibiotics, many countries are moving away from antibiotic growth promoters as a form of disease control. Nonetheless, relying solely on vaccinations could have hazardous consequences. Several natural occurring immunostimulants such as vitamin C, β-glucan and lactoferrin may serve as a supplemental treatment to vaccination (Sakai, 1999; Gopalakannan & Arul, 2006); since some of these immunostimulants possess the ability to stimulate humoral or cell-mediated immunity (Engstad *et al.*, 1992; Verlhac *et al.*, 1998; Gopalakannan & Arul, 2006). Chitin, a natural polysaccharide which is present in the exoskeleton of arthropods, have recently gained attention for its use as an immunostimulant (Esteban *et al.*, 2001; Cuesta *et al.*, 2003; Zaharoff *et al.*, 2007). Relative to size, chitin stimulates innate and adaptive immune responses (Lee *et al.*, 2008). Low doses of dietary chitin have been shown to have immunostimulating activity in fish (Esteban *et al.*, 2001; Gopalakannan & Arul, 2006). Even though most of the immunostimulatory research explored crustacean derived chitin in fish, it can be hypothesised that insect-derived chitin may also exhibit immunostimulatory activity in broilers.

Various biological and medicinal activities of extracts from house fly (*Musca domestica*) larvae have been reported, e.g., antitumor, antibacterial, antiviral, anti-allergic, anti-inflammatory as well as immunomodulatory activity (Meylaers *et al.*, 2004; Hou *et al.*, 2007; Chu *et al.*, 2011; Ai *et al.*, 2013). Injecting a protein-enriched fraction extracted house fly into mice increased the phagocytic function of their macrophages and increased the lymphoproliferation ability of their splenocytes (Ai *et al.*, 2013). The results suggest that fractions from larvae can enhance cellular immunity (T cell function) and might contain active components associated with T and B cell proliferation. Various compounds extracted from other insects also exhibited immunostimulating properties in mammals (Chernysh *et al.*, 2002; Ohta *et al.*, 2014, 2016). For example, a water-soluble polysaccharide extracted from melon fly pupae activates the mammalian innate immune response (Ohta *et al.*, 2014).

Due to nutritional value of fly larvae (Chapter 3) and their use in the bioconversion of waste, the use of fly larvae meal as an alternative protein source in animal feed has gained considerable attention in recent years. Since insects usually form part of the “natural” diet of poultry, several research studies investigated the effect of dietary larvae meal on growth performance and meat quality, with the majority of the studies showing no adverse effects on these parameters. Even though chitin particles and insect extracts show promise for immunostimulation, research on the immunomodulatory effects of dietary fly larvae meal in poultry diets is scarce.

When exploring any novel raw materials, it is important to consider food and feed safety aspects. A change in certain organ parameters can be an indication of certain chemical contaminants in the feed. For example, mycotoxins can result in gizzard erosion and can affect liver weight (D’Mello *et al.*, 1999). Differences in relative lymphoid organ weights in an environmentally controlled trial can also be an indication of immunocompetence as well as host exposure to toxins (Grasman, 2002).

Hence, the present research aims to study the effect of larvae meal from two fly species (*Chrysomya Chloropyga* (CC) and *Hermetia Illucens* (BSF)) as well as a commercial antimicrobial growth promotor (ZincBac), when included in the diets of broiler chickens, with respect to growth performance,

humoral, and cellular immune response. Traits such as liver colour, organ weights, gizzard erosion and intestinal pH were also determined to study possible modes of action for potentially enhanced performance.

4.2 Materials and methods

4.2.1 Animals and diets

One hundred and eight newly hatched female broiler chicks (Ross 308) used in this trial were obtained from a commercial hatchery. The birds were maintained in a temperature-controlled broiler house at Mariendahl experimental farm, Stellenbosch University, South Africa. Birds were provided with a commercial broiler starter diet for the first seven days. Treatments were set up according to the inclusion of either BSF or CC larvae meal at two inclusion levels (10% or 15%). On day seven, birds were weighed and individually caged in wire cages (0.45 m x 0.6 m). Each cage was provided with a nipple drinker and feeder containing the treatment diets. Cages were randomly divided into six treatment groups, with 18 replications/cages per treatment. Ethical approval to conduct the study was granted by the Research Ethics Committee: Animal Care and Use of Stellenbosch University, Stellenbosch (registration number SU-ACUD16-00043).

Dietary treatments were:

(i) **CON,** a maize-soya-fishmeal based control diet
(ii) **ZincBac**, a maize-soya-fishmeal based control diet supplemented with Zinc Bacitracin
(iii) **10%BSF**, *H. illucens* larvae meal included in the diet at a 10% inclusion level
(iv) **15%BSF**, *H. illucens* larvae meal included in the diet at a 15% inclusion level
(v) **10%CC**, *C. chloropyga* larvae meal included in the diet at a 10% inclusion level
(vi) **15%CC**, *C. chloropyga* larvae meal included in the diet at a 15% inclusion level

Iso-nitrogenous and iso-energetic experimental diets were formulated using feed formulation software (Winfeed V 2.8), conforming to the minimum nutrient requirements supplied by the Ross 308 broiler guide (Aviagen, 2014). Larvae meal starter diets were formulated by replacing fishmeal and partially replacing soya bean meal sources (full fat and oilcake) and vegetable oil in the control diets. Grower (Table 4.2) and finisher (Table 4.3) diets contained no fishmeal; therefore, the larvae meal only partially replaced the soya bean meal sources and vegetable oil. Diets were mixed at Mariendahl experimental farm. Individual body weight and feed intake were recorded on a weekly interval until slaughter at 42 days of age.

Table 4.1 Ingredient and calculated nutrient composition of trial starter diets

Ingredients	Units	CON[1]*	10%CC[2]	15%CC[3]	10%BSF[4]	15%BSF[5]
Maize	%	50.65	55.66	60.15	49.42	50.65
Soya bean (Full fat)	%	16.00	11.70	--	14.90	5.06
Soya bean (46%)	%	22.86	18.10	20.35	21.72	25.68
Fishmeal	%	3.00	--	--	--	--
L-lysine (HCl)	%	0.27	--	--	0.08	0.10
DL methionine	%	0.27	0.19	0.14	0.18	0.13
L-threonine	%	0.12	--	--	--	--
Premix	%	0.25	0.25	0.25	0.25	0.25
Limestone	%	1.17	1.20	0.16	0.53	0.14
Salt	%	0.30	0.23	0.24	0.29	0.25
Mono-calcium phosphate	%	1.32	1.67	1.78	1.64	1.73
Sunflower oil	%	3.84	1.00	1.00	1.00	1.00
Hermetia illucens larvae meal	%	--	--	--	10.00	15.00
Chrysomya chloropyga larvae meal	%	--	10.00	15.00	--	--
Calculated nutrient composition						
Dry matter	%	89.01	89.24	89.31	88.78	88.98
AMEn	MJ/kg	13.30	13.30	13.30	13.30	13.30
Crude protein	%	228.00	228.39	228.39	228.00	228.00
Crude fibre	%	32.82	36.74	36.88	40.49	42.54
Crude fat	%	93.15	78.94	71.51	98.05	100.59
Lysine	%	15.00	13.34	13.56	13.20	13.20
Methionine	%	6.35	5.92	5.76	5.43	5.02
Cysteine	%	3.45	3.88	4.04	4.37	4.78
Methionine + Cysteine	%	9.80	9.80	9.80	9.80	9.80
Threonine	%	10.00	8.99	9.02	9.10	9.20
Tryptophan	%	2.74	2.30	2.00	2.98	3.03
Arginine	%	15.20	15.79	15.82	15.94	16.08
Isoleucine	%	10.37	10.17	9.94	10.58	10.58
Valine	%	11.17	11.44	11.58	11.81	12.15
Calcium	%	9.00	9.00	9.00	9.00	9.00
Total Phosphorous	%	7.20	7.63	7.79	7.90	8.21
Available phosphorous	%	4.50	4.50	4.50	4.50	4.50
Sodium	%	1.60	1.60	1.60	1.60	1.60
Chloride	%	2.63	3.00	3.18	2.36	2.22
Potassium	%	8.98	8.29	7.52	9.27	9.01

[1]CON = control diet; [2]10%CC = diet containing 10% *C. chloropyga* larvae meal; [3]15%CC = diet containing 15% *C. chloropyga* larvae meal; [4]10%BSF = diet containing 10% *H. illucens* larvae meal; [5]15%BSF = diet containing 15% H. *illucens* larvae meal; *The same control diet was used for the ZincBac diet, with an addition of 50mg/kg Zinc Bacitracin
[6]AMEn = Nitrogen-corrected apparent metabolisable energy
*The same control diet was used for the ZincBac diet, with an addition of 50mg/kg Zinc Bacitracin

Table 4.2 Ingredient and calculated nutrient composition of trial grower diets

Ingredients	Units	CON*	10%CC	15%CC	10%BSF	15%BSF
Maize	%	50.667	53.680	51.944	57.559	59.113
Soya bean (Full fat)	%	20.000	24.190	17.270	18.791	7.932
Soya bean (46%)	%	18.895	9.040	13.030	10.402	15.136
L-lysine (HCl)	%	0.278	0.024	--	0.379	0.415
DL methionine	%	0.330	0.179	0.092	0.241	0.190
L-threonine	%	1.316	--	--	0.099	0.091
Premix	%	0.200	0.200	0.200	0.200	0.200
Limestone	%	1.399	1.483	1.522	0.872	0.612
Salt	%	0.215	0.093	0.004	0.184	0.163
Mono-calcium phosphate	%	1.347	1.066	0.936	1.039	0.897
Sodium bicarbonate	%	0.210	0.046	0.001	0.233	0.251
Sunflower oil	%	5.143	--	--	--	--
Hermetia illucens larvae meal	%	--	--	--	10.00	15.00
Chrysomya chloropyga larvae meal	%	--	10.00	15.00	--	--
Calculated nutrient composition						
Dry matter	%	89.088	88.682	88.895	88.469	88.513
AMEn	MJ/kg	13.000	13.000	13.000	13.000	13.000
Crude protein	%	21.900	22.566	23.439	20.525	20.433
Crude fibre	%	3.159	3.700	3.850	3.753	3.893
Crude fat	%	11.000	8.951	8.814	9.651	9.688
Lysine	%	1.369	1.410	1.546	1.370	1.368
Methionine	%	0.636	0.592	0.562	0.570	0.532
Cysteine	%	0.358	0.432	0.472	0.439	0.479
Methionine + Cysteine	%	0.994	1.024	1.034	1.010	1.011
Threonine	%	2.106	0.923	1.005	0.896	0.895
Tryptophan	%	0.244	0.229	0.231	0.248	0.255
Arginine	%	1.407	1.623	1.777	1.368	1.368
Isoleucine	%	0.938	1.032	1.111	0.898	0.893
Leucine	%	1.817	1.981	2.084	1.798	1.791
Histidine	%	0.566	0.707	0.788	0.556	0.553
Phenylalanine	%	0.964	1.146	1.261	0.918	0.903
Tyrosine	%	0.785	0.848	0.921	0.749	0.762
Phenylalanine + Tyrosine	%	1.749	1.994	2.182	1.667	1.665
Valine	%	1.042	1.195	1.302	1.065	1.092
Ash	%	4.113	4.341	4.498	4.304	4.407
Calcium	%	0.840	0.840	0.840	0.840	0.840
Total Phosphorous	%	0.717	0.690	0.675	0.680	0.657
Available phosphorous	%	0.420	0.420	0.420	0.420	0.420
Sodium	%	0.160	0.160	0.160	0.160	0.160
Chloride	%	0.220	0.220	0.220	0.220	0.220
Potassium	%	0.875	0.831	0.831	0.779	0.740

[1]CON = control diet; [2]10%CC = diet containing 10% *C. chloropyga* larvae meal; [3]15%CC = diet containing 15% *C. chloropyga* larvae meal; [4]10%BSF = diet containing 10% *H. illucens* larvae meal; [5]15%BSF = diet containing 15% *H. illucens* larvae meal; *The same control diet was used for the ZincBac diet, with an addition of 50mg/kg Zinc Bacitracin
[6]AMEn = Nitrogen-corrected apparent metabolisable energy
*The same control diet was used for the ZincBac diet, with an addition of 50mg/kg Zinc Bacitracin

Table 4.3 Ingredient and calculated nutrient composition of trial finisher diets

Ingredients	Units	CON*	10%CC	15%CC	10%BSF	15%BSF
Maize	%	47.243	56.102	59.447	48.149	51.265
Soya bean (Full fat)	%	30.000	20.000	9.952	20.000	12.904
Soya bean (46%)	%	15.439	10.025	12.309	17.655	18.124
L-lysine (HCl)	%	--	--	--	--	0.071
DL methionine	%	0.121	0.020	--	0.049	0.041
L-threonine	%	--	--	--	--	--
Premix	%	0.150	0.150	0.150	0.150	0.150
Limestone	%	1.657	1.794	1.861	1.115	0.859
Salt	%	0.274	0.096	0.002	0.255	0.231
Mono-calcium phosphate	%	1.647	1.392	1.268	1.352	1.207
Sodium bicarbonate	%	0.085	0.044	0.011	0.123	0.147
Sunflower oil	%	3.382	0.377	--	1.151	--
Hermetia illucens larvae meal	%	--	--	--	10.000	15.000
Chrysomya chloropyga larvae meal	%	--	10.000	15.000	--	--
Calculated nutrient composition						
Dry matter	%	88.93	88.66	88.68	88.89	88.78
AMEn	MJ/kg	13.30	13.30	13.30	13.30	13.30
Crude protein	%	22.34	22.50	22.34	22.95	22.50
Crude fibre	%	3.46	3.57	3.58	3.98	4.14
Crude fat	%	10.92	8.66	7.73	10.77	10.35
Lysine	%	1.28	1.33	1.37	1.29	1.28
Methionine	%	0.45	0.42	0.44	0.42	0.42
Cysteine	%	0.38	0.42	0.44	0.47	0.51
Methionine + Cysteine	%	0.84	0.85	0.89	0.89	0.92
Threonine	%	0.87	0.89	0.91	0.92	0.91
Tryptophan	%	0.27	0.22	0.20	0.29	0.29
Arginine	%	1.55	1.55	1.58	1.61	1.58
Isoleucine	%	1.03	0.99	0.98	1.06	1.03
Leucine	%	1.94	1.92	1.93	1.98	1.95
Histidine	%	0.62	0.68	0.72	0.63	0.62
Phenylalanine	%	1.05	1.10	1.14	1.06	1.03
Tyrosine	%	0.84	0.82	0.83	0.88	1.95
Phenylalanine + Tyrosine	%	1.89	1.92	1.97	1.94	0.62
Valine	%	1.13	1.15	1.18	1.22	1.89
Ash	%	4.62	4.52	4.51	4.92	1.22
Calcium	%	1.00	1.00	1.00	1.00	1.00
Total Phosphorous	%	0.82	0.76	0.73	0.79	0.76
Available phosphorous	%	0.50	0.50	0.50	0.50	0.50
Sodium	%	0.15	0.16	1.16	0.16	0.16
Chloride	%	0.22	0.22	0.22	0.22	0.22
Potassium	%	0.85	0.79	0.73	0.91	0.85

[1]CON = control diet; [2]10%CC = diet containing 10% *C. chloropyga* larvae meal; [3]15%CC = diet containing 15% *C. chloropyga* larvae meal; [4]10%BSF = diet containing 10% *H. illucens* larvae meal; [5]15%BSF = diet containing 15% *H. illucens* larvae meal; *The same control diet was used for the ZincBac diet, with an addition of 50mg/kg Zinc Bacitracin
[6]AMEn = Nitrogen-corrected apparent metabolisable energy
*The same control diet was used for the ZincBac diet, with an addition of 50mg/kg Zinc Bacitracin

4.2.2 Immunisation with Sheep red blood cells (SRBC)

Blood collected in EDTA tubes from the brachial vein of Dohne Merino sheep was used for the harvesting of sheep red blood cells (SRBC). Blood was centrifuged (580 x g for ten minutes at 4°C) and the serum was discarded by means of a pipette. The remaining red blood cells were washed with phosphate-buffered solution (PBS). These steps were repeated three times until the red blood cells were free from any serum and debris. The SRBC were diluted with PBS to get a concentration of 2% SRBC. The SRBC solution was stored at -4°C for maximum 24 hours until immunisation of the broilers took place.

On day 21 post-hatching, blood of all the broilers was collected to ensure that no agglutination response against SRBC can be detected in the serum before immunisation. Subsequently, the birds were immunised intramuscularly with 0.25 ml of the 2% SRBC solution. To determine the secondary immune response, the birds were immunised again with freshly prepared SRBC on day 28. Blood samples were collected *via* the wing web on day 28 (for primary humoral immune response), 35 and 42 (for secondary response). For serum collection, blood was kept at room temperature for two hours and centrifuged for ten minutes at 4°C. Serum was stored at -20°C until further analysis.

4.2.3 Haemagglutination assay

Serum samples were placed in a water bath for 30 minutes at 56°C to inactivate the compliment. A volume of 50 µl PBS was added to each well of a 96 well U-shaped bottom microplate. A volume of 50 µl serum sample was added to the first well of each row. Serial two-fold dilutions were made of each sample into the remaining 11 wells of the row. Fifty µl of freshly prepared SRBC suspension (2%) was added to each well. Total antibody titres (HA titre) were read after 30 minutes incubation at 37°C. Titers were expressed as Log_2 of the reciprocal of the highest dilution giving visible agglutination. The titre measured the activity of total (IgM and IgG) haemagglutinating antibodies that were produced in response to immunisation with SRBC

4.2.4 Toe web thickness response to PHA-P (lymphoproliferative response)

The lymphoproliferative response to PHA-P, an indicator of T cell-mediated immune responsiveness in animals, was assessed on day 32 (25 days after the commencement of treatment diets) using the phytohaemagglutinin (PHA-P) mitogen test described by Corrier & DeLoach (1990). Briefly, the toe web between the third and fourth digits of the left and right foot was measured using a thickness meter with an accuracy of 0.01 mm. Immediately after measurement, a dose of 0.1 ml of 1 mg PHA-P (L8754, Sigma Aldrich, St. Louis, MO, USA) dissolved in 100 µl phosphate-buffered saline (PBS) was injected into the toe web of the left foot, while the right-wing web received a control injection of 0.1 ml sterile PBS alone. The toe web swelling was measured 24 hours after injection.

The wing web swelling reactions to PHA-P were calculated using equation 4.1:

Equation 4.1

$$\text{Index} = (\text{mm post PHA injection} - \text{mm pre PHA injection}) - (\text{mm post PBS injection} - \text{mm pre PBS injection})$$

4.2.5 Haematological parameters

On day 38, blood was collected from each bird *via* the brachial vein into a 1 ml K_2-EDTA BD Vacutainer® blood collection tube (Becton, Dickinson and Company, New Jersey, USA). Automated full blood counts, erythrocyte counts and their related parameters, as well as total leukocyte count were measured using the Celldyne 3700CS haematology analyser (Abbott Diagnostics, Illinois, USA).

4.2.6 Organ parameters

All the birds were slaughtered at 42 days of age by means of cervical dislocation. The organs (spleen, bursa of Fabricius, heart, liver, and gizzard) were excised from the fresh carcass and weighed using a Mettler PC 4400 laboratory scale (Mettler-Toledo, Switzerland) to determine relative organ weight. The gizzard was cut open longitudinally, rinsed and scored for gizzard erosion on an ordinal scale as described in Table 4.4. The pH of the proventriculus, duodenum, jejunum, ileum, and cecum were measured using a calibrated portable Crison pH25 meter (Alella, Barcelona) by inserting the pH electrode into the cut end of the area in the digestive tract. The probe was thoroughly rinsed with distilled water between readings. The liver colour was measured in triplicate with a colour-guide 45°/0° colourimeter (Cat no. 6805, BYK-Gardner, USA). These measurements were used to determine the colour of the liver in terms of CIE L* (brightness), CIE a* (red-green range) and CIE b*-values (blue-yellow range). Positive a* values are a measure of redness and negative a* values are a measure of greenness. Positive b* values are a measure of yellowness, and negative b* values indicate blueness.

Table 4.4 Gizzard Erosion scoring description (Sugahara *et al.*, 1988)

Score	Description
0	Healthy gizzard with no erosion
1	Light erosion - healthy gizzard with small local aberrations
2	Modest erosion - large aberrations or more than three aberrations
3	Severe erosion - severely affected gizzards with haemorrhaging or loss of keratin
4	Extreme erosion - severe damage with perforation of the gizzard

4.3 Results

4.3.1 Production parameters

There were no differences for live weight at placement on seven days of age (data not shown). Live weight gain was significantly affected by treatment on day 14 and 21 (Table 4.5). Up until day 14, chicks in the 15%CC treatment group had the highest live weight gain compared to the CON and 10%BSF group. During this period, there were no significant differences for feed intake between treatment groups; however, the increased live weight gain for the 15%CC treatment group resulted in an improved FCR when compared to the 10%BSF group. Birds in the 15%BSF treatment group had a significantly higher live weight gain from day seven till day 21 when compared to the 10%BSF group. Even though birds in the 15%BSF treatment group had a higher feed intake than ZincBac birds up to day 28, the FCR for this period was unaffected. There were no differences for live weight gain from day seven till day 35, but there was a tendency (P=0.053) towards an increased intake for the 15%BSF group, resulting in a significantly higher FCR for the 15%BSF treatment group when compared to CON treatment group. The FCR for all the other groups were intermediate to the CON and 15%BSF group. At 42 days of age (35 days of receiving the treatment diets), there were no significant differences for total live weight gain, feed intake or feed conversion ratios between treatment groups.

4.3.2 Antibody response (Haemagglutination titre)

Antibody response to SRBC as measured in total IgM and IgG levels are depicted in Figure 4.1. Total antibody titres were measured seven days post-primary injection (day 21) and seven and 14 days post-secondary injection (day 35 and 42). For primary antibody response, birds in the 10%CC and 10%BSF group exhibited a higher antibody response compared to birds in the CON and ZincBac group, with the 15%CC and 15%BSF group being intermediate to these groups (P = 0.004). Seven days after the second injection with SRBC, birds in the 10%CC and 15%CC group had a higher antibody response compared to birds in the ZincBac group, but only birds in the 10%CC group differed significantly from the CON group (P = 0.017). From day 35 to 42, there was a more severe increase in antibody titres in the CON, ZincBac, 15%BSF and 15%CC chickens, owing to no significant differences between any of the treatment groups (P = 0.115).

4.3.3 Lymphoproliferative response to PHA-P (Toe web thickness index)

The response to PHA-P, a mitogen which induces proliferation in T-lymphocytes, was measured on 32 days of age in all the chickens (Figure 4.2). The response measured 24 hours after PHA-P injection was higher in all the treatment groups that received dietary larvae meal compared to the CON treatment group. Birds in the 10%CC and 10%BSF group had a higher response compared to the ZincBac group, while birds in the 15%CC and 15%BSF group were intermediate to the 10%CC and ZincBac groups.

Table 4.5 The effect of dietary inclusion of dietary larvae meal or Zinc Bacitracin on the production parameters of broiler chickens (mean ± s.e.)

	[1]Treatments						
	CON	ZincBac	10%CC	15%CC	10%BSF	15%BSF	P- value
Day 7-14							
BWG	239^{b} ± 5	246^{ab} ± 5	246^{ab} ± 7	264^{a} ± 5	228^{b} ± 7	242^{ab} ± 3	0.001
FI	307 ± 6	311 ± 7	319 ± 8	328 ± 8	305 ± 8	312 ± 4	0.172
FCR	1.29^{ab} ± 0.02	1.27^{ab} ± 0.02	1.31^{ab} ± 0.02	1.24^{b} ± 0.02	1.35^{a} ± 0.02	1.29^{ab} ± 0.01	0.026
Day 7-21							
BWG	628^{ab} ± 13	614^{ab} ± 9	612^{ab} ± 13	631^{ab} ± 10	592^{b} ± 14	642^{a} ± 7	0.025
FI	818 ± 15	819 ± 12	839 ± 15	850 ± 10.8	841 ± 16.7	869 ± 8	0.062
FCR	1.31^{b} ± 0.03	1.34^{b} ± 0.01	1.37^{ab} ± 0.02	1.35^{b} ± 0.02	1.42^{a} ± 0.02	1.35^{b} ± 0.01	0.001
Day 7-28							
BWG	1120 ± 22	1088 ± 20	1135 ± 28	1134 ± 27	1112 ± 24	1176 ± 18	0.179
FI	1619^{ab} ± 34	1599^{b} ± 30	1695^{ab} ± 43	1694^{ab} ± 36	1661^{ab} ± 36	1761^{a} ± 26	0.021
FCR	1.45 ± 0.02	1.47 ± 0.02	1.50 ± 0.03	1.48 ± 0.01	1.50 ± 0.02	1.49 ± 0.01	0.315
Day 7-35							
BWG	1801 ± 31	1784 ± 25	1818 ± 62	1805 ± 32	1776 ± 33	1814 ± 25	0.971
FI	2853 ± 55	2893 ± 60	2996 ± 80	2961 ± 54	2903 ± 50	3101 ± 57	0.053
FCR	1.58^{b} ± 0.02	1.63^{ab} ± 0.02	1.64^{ab} ± 0.03	1.64^{ab} ± 0.02	1.64^{ab} ± 0.02	1.70^{a} ± 0.02	0.007
Day 7-42							
BWG	2371 ± 45	2373 ± 26	2411 ± 87	2408 ± 31	2366 ± 44	2394 ± 38	0.965
FI	4108 ± 75	4196 ± 74	4215 ± 111	4210 ± 64	4119 ± 77	4365 ± 66	0.209
FCR	1.73 ± 0.01	1.78 ± 0.02	1.75 ± 0.03	1.75 ± 0.02	1.74 ± 0.02	1.79 ± 0.02	0.228
Mortality	0	0	0	0	0	0	1.000

[1] CON = Broilers receiving control diet; ZincBac = Broilers receiving control diet supplemented with Zinc Bacitracin; 10%CC and 15%CC = broilers receiving diets containing 10% or 15% *C. chloropyga* larvae meal, respectively; 10%BSF and 15%BSF = broilers receiving diets containing 10% or 15% *H. illucens* larvae meal, respectively.

[a,b] Means within rows with different superscripts differ significantly (P <0.05)

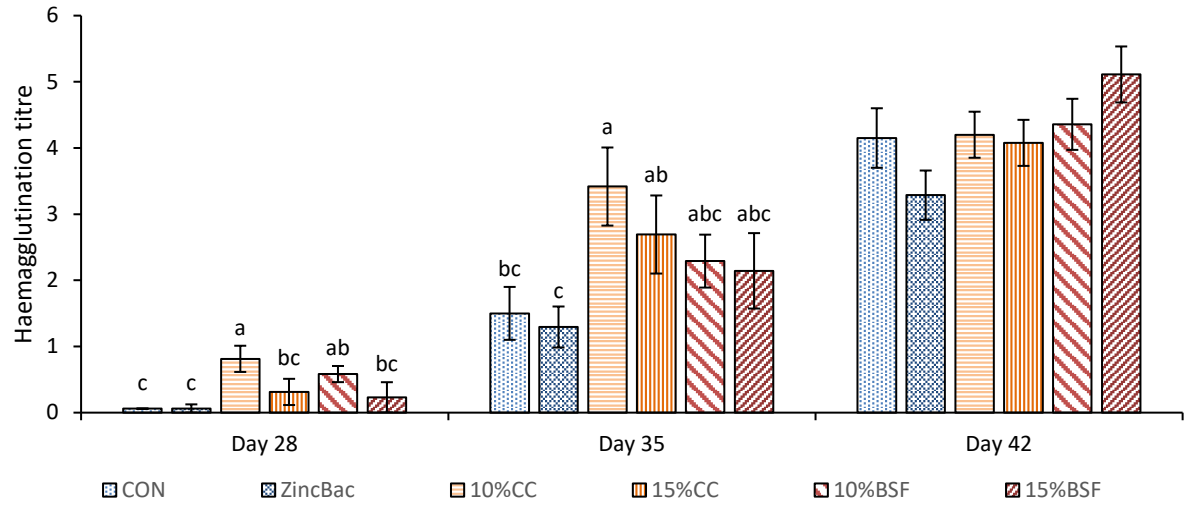

Figure 4.1 Mean antibody titre response to sheep red blood cell injections on day 21 and 28. The response was measured on day 28, 35 and 42. Treatment groups were: CON = Broilers receiving control diet; ZincBac = Broilers receiving control diet supplemented with Zinc Bacitracin; 10%CC and 15%CC = broilers receiving diets containing 10% or 15% *C. chloropyga* larvae meal, respectively; 10%BSF and 15%BSF = broilers receiving diets containing 10% or 15% *H. illucens* larvae meal, respectively.
[a,b,c] Bars with different superscripts differ significantly (P <0.05). Bars within a time period with no superscripts do not differ significantly. Error bars represent standard error of mean.

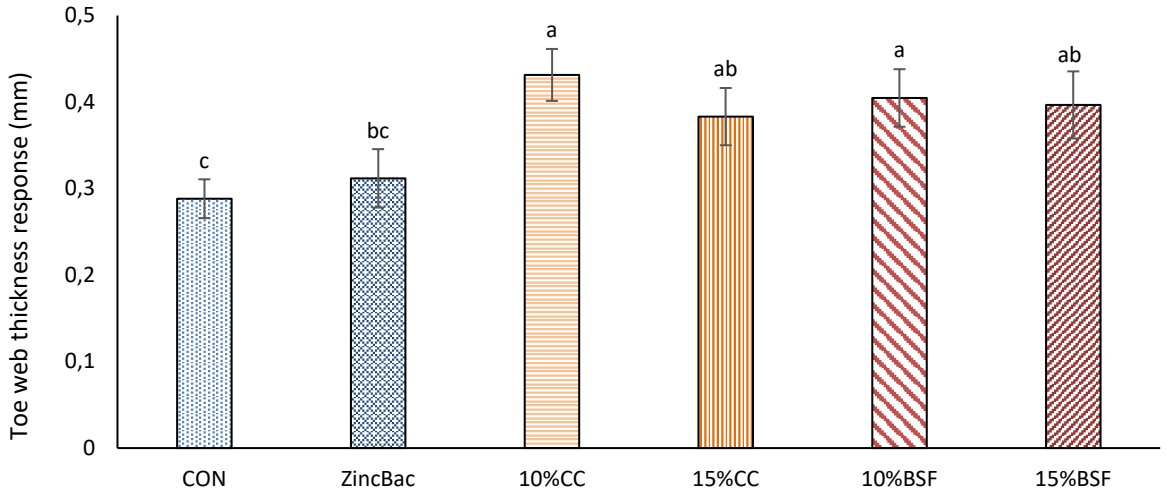

Figure 4.2 Mean toe web thickness response to injection with phytohemagglutinin (PHA-P) on day 32. Treatment groups were: CON = Broilers receiving control diet; ZincBac = Broilers receiving control diet supplemented with Zinc Bacitracin; 10%CC and 15%CC = broilers receiving diets containing 10% or 15% *C. chloropyga* larvae meal, respectively; 10%BSF and 15%BSF = broilers receiving diets containing 10% or 15% *H. illucens* larvae meal, respectively.
[a,b,c] Bars with different superscripts differ significantly (P <0.05). Error bars represent standard error of the mean.

4.3.4 Haematological parameters

Haematological parameters were overall comparable, except for mean corpuscular volume (MCV) and mean cell haemoglobin concentrations (MCHC). Birds in the 10%CC group had significantly lower MCV values compared to the CON and 15%CC group while birds in the 15%CC group had significantly higher MCV values compared to the ZincBac, 10%BSF, 15%BSF and 10%CC group. The MCHC values were significantly lower in the 15%CC group compared to the 10%CC group

Table 4.6 The effect of dietary inclusion of dietary larvae meal or oxytetracycline on the haematological parameters of *Salmonella* Enteritidis infected broiler chickens (mean ± s.e)

	[1]Treatments						
	CON	ZincBac	10%CC	15%CC	10%BSF	15%BSF	*P-value*
WBC	22.2 ± 0.6	22.0 ± 0.6	22.2 ± 0.8	21.6 ± 1.0	22.3 ± 0.8	21.9 ± 0.5	0.992
RBC	2.47 ± 0.03	2.49 ± 0.05	2.53 ± 0.08	2.48 ± 0.07	2.47 ± 0.05	2.46 ± 0.04	0.963
HGB	14.2 ± 0.2	14.1 ± 0.3	14.4 ± 0.4	14.3 ± 0.4	14.0 ± 0.2	14.1 ± 0.2	0.916
PCV	20.9 ± 0.4	20.82 ± 0.4	20.9 ± 0.6	21.2 ± 0.6	20.3 ± 0.5	20.63 ± 0.3	0.884
MCV	84.4^{ab} ± 0.7	83.5^{bc} ± 0.6	82.4^{c} ± 0.5	85.5^{a} ± 0.7	82.5^{bc} ± 0.65	83.7^{abc} ± 0.7	0.021
MCH	57.3 ± 0.4	56.5 ± 0.5	56.9 ± 0.3	57.7 ± 0.4	56.9 ± 0.4	56.9 ± 0.3	0.380
MCHC	67.9^{bc} ± 0.4	67.7^{c} ±0.5	69.2^{a} ± 0.3	67.5^{c} ± 0.4	69.0^{ab} ± 0.3	68.1^{abc} ± 0.4	0.011
RDW	22.2 ± 0.6	12.1 ± 0.16	12.0 ± 0.16	11.8 ± 0.17	12.2 ± 0.18	12.0 ± 0.16	0.821

[1] CON = Broilers receiving control diet; ZincBac = Broilers receiving control diet supplemented with Zinc Bacitracin; 10%CC and 15%CC = broilers receiving diets containing 10% or 15% *C. chloropyga* larvae meal, respectively; 10%BSF and 15%BSF = broilers receiving diets containing 10% or 15% *H. illucens* larvae meal, respectively.
WBC = Leukocyte count; RBC = Erythrocyte count; HGB = Haemoglobin; MCV = Mean corpuscular volume; RDW = Erythrocyte distribution width; PCV = packed cell volume / haematocrit; MCH = mean cell haemoglobin, MCHC = Mean cell haemoglobin concentrations
[a,b,c] Means within rows with different superscripts differ significantly (P <0.05)

4.3.5 Organ parameters

Relative organ weight is an expression of organ weight as a percentage of body weight. The inclusion of 10% CC larvae meal significantly increased gizzard weight compared to the ZincBac treatment group (Table 4.7), but treatments had no effect on any of the other organ weighs. The gizzard erosion score (Table 4.8), pH of the different parts of the digestive tract (Table 4.9), and liver colour (Table 4.9) were unaffected by dietary treatments.

Table 4.7 The effect of dietary inclusion of dietary larvae meal or Zinc Bacitracin the relative organ weights of broiler chickens (mean ± s.e.)

	[1]Treatments						
	CON	ZincBac	10%CC	15%CC	10%BSF	15%BSF	*P-value*
Bursa	0.11 ± 0.01	0.11 ± 0.01	0.14 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.13 ± 0.01	0.367
Spleen	0.16 ± 0.01	0.16 ± 0.01	0.18 ± 0.02	0.18 ± 0.01	0.17 ± 0.01	0.19 ± 0.01	0.193
Spleen/ bursa	1.58 ± 0.09	1.57 ± 0.11	1.45 ± 0.11	1.78 ± 0.21	1.66 ± 0.12	1.53 ± 0.11	0.639
Heart	0.53 ± 0.02	0.52 ± 0.01	0.49 ± 0.02	0.49 ± 0.02	0.50 ± 0.01	0.49 ± 0.02	0.373
Liver	1.97 ± 0.05	2.01 ± 0.05	2.03 ± 0.17	2.04 ± 0.10	1.99 ± 0.08	2.06 ± 0.07	0.955
Gizzard	1.29^{ab} ± 0.04	1.24 ^{b} ± 0.03	1.38 ^{a} ± 0.03	1.37 ^{a} ± 0.04	1.32 ^{ab} ± 0.03	1.31 ^{ab} ± 0.03	0.048

[1] CON = Broilers receiving control diet; ZincBac = Broilers receiving control diet supplemented with Zinc Bacitracin; 10%CC and 15%CC = broilers receiving diets containing 10% or 15% *C. chloropyga* larvae meal, respectively; 10%BSF and 15%BSF = broilers receiving diets containing 10% or 15% *H. illucens* larvae meal, respectively.
[a,b] Means within rows with different superscripts differ significantly (P <0.05)

Table 4.8 The effect of dietary inclusion of dietary larvae meal or Zinc Bacitracin on gizzard erosion scores of broiler chickens

[1]Treatment	**Gizzard score**				
	0	**1**	**2**	**3**	**4**
CON	11	7	0	0	0
ZincBac	15	3	0	0	0
10%CC	12	6	0	0	0
15%CC	12	5	1	0	0
10%BSF	10	7	1	0	0
15%BSF	13	2	3	0	0
Chi-Square *P* value	0.562				

[1] CON = Broilers receiving control diet; ZincBac = Broilers receiving control diet supplemented with Zinc Bacitracin; 10%CC and 15%CC = broilers receiving diets containing 10% or 15% *C. chloropyga* larvae meal, respectively; 10%BSF and 15%BSF = broilers receiving diets containing 10% or 15% *H. illucens* larvae meal, respectively.

Table 4.9 The effect of dietary inclusion of dietary larvae meal or Zinc Bacitracin on the pH of various parts of the digestive tract and liver colour of broiler chickens (mean ± s.e)

	[1]Treatments						
	CON	ZincBac	10%CC	15%CC	10%BSF	15%BSF	*P-value*
Digestive tract pH							
Proventriculus	3.66 ± 0.20	3.56 ± 0.19	3.77 ± 0.24	3.89 ± 0.14	3.78 ± 0.21	3.89 ± 0.12	0.802
Duodenum	6.02 ± 0.23	6.06 ± 0.09	6.02 ± 0.15	5.99 ± 0.14	6.10 ± 0.11	5.90 ± 0.12	0.958
Jejunum	6.51 ± 0.05	6.44 ± 0.04	6.37 ± 0.06	6.38 ± 0.07	6.39 ± 0.04	6.28 ± 0.06	0.072
Ileum	6.87 ± 0.06	7.03 ± 0.08	7.05 ± 0.14	7.01 ± 0.07	7.00 ± 0.07	6.82 ± 0.06	0.236
Ceca	6.73 ± 0.08	6.70 ± 0.06	6.82 ± 0.09	6.65 ± 0.09	6.59 ± 0.12	6.68 ± 0.07	0.588
Liver colour							
L	34.16 ± 0.7	35.49 ± 0.7	36.70 ± 0.9	36.02 ± 0.8	35.46 ± 0.79	35.97 ± 0.7	0.299
a	10.37 ± 0.6	9.57 ± 0.8	9.03 ± 0.7	10.31 ± 0.7	9.82 ± 0.68	9.14 ± 0.6	0.658
b	9.27^{b} ± 0.8	10.22^{b} ± 0.7	12.40^{a} ± 0.8	9.85^{b} ± 0.7	9.25^{b} ± 0.55	10.21^{b} ± 0.9	0.051

[1] CON = Broilers receiving control diet; ZincBac = Broilers receiving control diet supplemented with Zinc Bacitracin; 10%CC and 15%CC = broilers receiving diets containing 10% or 15% *C. chloropyga* larvae meal, respectively; 10%BSF and 15%BSF = broilers receiving diets containing 10% or 15% *H. illucens* larvae meal, respectively.
[a,b] Means within rows with different superscripts differ significantly (P <0.05)

4.4 Discussion

4.4.1 Production parameters

The inclusion of high levels of larvae meal had a positive effect on production parameters up to day 21 (14 days after commencement of the trial). The favourable effects diminished with time, leading to a poorer FCR in birds receiving 15% BSF meal up to day35. These results are in agreement with Dabbou *et al*. (2018), who reported no differences in FCR in birds receiving 0%, 5% 10% or 15% BSF larvae meal for ten days, but FCR was poorer for birds receiving 15% BSF meal at the end of the trial period of 35 days. On the other hand, Onsongo *et al*. (2018) and Uushona, (2015) observed no negative effects on growth or FCR when 15% BSF larvae or prepupae were added to broiler diets. Van der Merwe (2018) also fed broilers diets containing CC larvae meal. When using a 10% inclusion rate, he observed an improvement in weight gain and FCR during the grower phase, as well as an increase in slaughter weight. However, a 5% and 15% inclusion rate resulted in production parameters similar to the control group. The reason for the poor FCR during the finisher period for the 15%BSF treatment birds in this study is unclear. Since chitin level in the diet has a negative effect on protein and lipid digestibility (Hansen *et al*., 2010; Karlsen *et al*., 2017) it can be argued that the higher indigestible chitin content for the 15%BSF group, compared to the 10%BSF and CON group, may be the reason for this phenomenon. However, the chitin levels in the larvae meal sources in this study were not determined to prove this hypothesis.

4.4.2 Haemagglutination response (humoral or antibody response)

The effects of larvae meal on humoral immunity were tested by immunising the chicks with sheep red blood cells (SRBC), a non-pathogenic antigen, with the intention of provoking the production of antibodies. Immunisation with SRBC elicited a stronger response in broilers receiving larvae meal. Several factors in larvae meal suggest that it may exhibit a positive response on humoral immunity. For example, BSF larvae contains polyphenols (Janssen *et al*., 2019) and chitin, which are immunoglobulin stimulators (Koide, 1998; Taira *et al*., 2015). Not only do chitosan increase IgG in piglets (Li *et al*., 2013), it also increases the humoral and cell-mediated response in piglets (Li *et al*., 2013) and mice (Zaharoff *et al*., 2007). Although data on the effect of dietary fly larvae meal on the humoral immune response is scarce, the dietary inclusion of other insect meal sources revealed positive or neutral effects on immunoglobulin concentration in the studied animals. The inclusion of mealworm and super mealworm meal fermented in probiotics, increased immunoglobulins in broilers challenged with *Salmonella* (Islam & Yang, 2017), whereas the inclusion of mealworms had no effect on the general immunoglobulin concentration in unchallenged pigs (Jin *et al*., 2016). On the other hand, when mealworms were added to the diets of catfish, the expression of immunoglobulin-M (IgM) in the liver increased together with an increment of the transcriptional levels of genes for IgM in the spleen (Su *et al*., 2017). It is possible dietary insect meal only causes a spike in the synthesis of immunoglobulins when animals are being challenged, explaining immune response differences between studies.

4.4.3 Lymphoproliferative response

Phytohemagglutinin-P, a T-cell mitogen, induces proliferation in T-lymphocytes. Injection of PHA-P at a selected site in poultry serves as an inducer of localised in vivo T-lymphoproliferative response and has been shown to be a reliable indicator of in vivo cellular immunity in poultry (Goto *et al.*, 1978; McCorckle *et al.*, 1980). The exact physiological mechanism by which the inclusion of either larvae meal sources increased the T-lymphocyte immune function in the present study is difficult to ascertain. Protein malnutrition is known to impair T-lymphocyte-dependant immune functions (Lochmiller *et al.*, 2018). However, if protein availability was the factor responsible for the impaired immune response, the bodyweight of the CON treatment group would have been negatively affected as well.

The skin response after PHA-P injection reflects a complex series of physiological processes such as cellular proliferation, the release of chemical mediators, and changes in vascularity. Intradermal injections of PHA-P elicit macrophage infiltration, accumulation of lymphocytes, basophils and eosinophils (McCorckle *et al.*, 1980; Klaus, 1996). T-lymphocytes are produced in the thymus, with the most abundant subsets among these cells, being, CD4+, CD5+ and CD8+ cells. The CD4+ cells participate in the first phase of the skin-swelling response by activating local cell populations, mainly basophils and macrophages, resulting in exudation of plasma and oedema at the site of injection (Klaus, 1996). Chitosan, a linear polysaccharide derived from chitin, has been shown to enhance CD4+ proliferation (Zaharoff *et al.*, 2007), while chitin and other insect-derived substances (i.e. Dipterose) stimulates macrophages (Lee *et al.*, 2008; Ohta *et al.*, 2014). Hence, the above-mentioned factors could explain the heightened T-cell mediated response in the larvae meal fed broilers.

4.4.4 Haematological parameters

Haematological parameters can be used to evaluate the health status of the animal. The erythrocyte count (RBC) and packed cell volume/haematocrit (PCV) decreases during severe infection or cecal haemorrhage (Natt & Herrick, 1955; Conway *et al*., 2013); while elevated leukocyte count WBC is an indication of bacterial infection or parasitism. Then again, WBC below the normal range can be an indication of allergies or malnutrition (Cavanaugh, 2003). Haematological values do not only a give an indication of the health status of the animal, but it is also sensitive to the nutritional status of the diet, with mean corpuscular volume (MCV) and mean cell haemoglobin (MCH) being most sensitive to dietary changes (Hackbarth, 1983). The dietary inclusion of larvae meal had no effect on WBC, RBC, haemoglobin (HGB), erythrocyte distribution width (RDW), PCV or MCH values in the current study. Similarly, other studies reported no effects on these traits after the inclusion of BSF larvae (Iaconisi *et al*., 2017), yellow mealworm larvae (Bovera *et al*., 2015) or silkworm larvae meal (Anggraeni *et al*., 2016) to poultry diets.

A significantly lower mean MCV, an indication of smaller red blood cell size, was observed in the 10%CC treatment group when compared to the CON and 15%CC treatment group. Low MCV values are often an indication of microcytic anaemia, typically a result of iron deficiency. The lower MCV level in 10%CC chickens was unexpected. Even though reported iron levels in BSF larvae ranged between medium (110 mg/kg) to high (1568 mg/kg) levels (Haasbroek, 2016; Spranghers *et al*., 2017), reported iron levels in CC larvae meal ranged between 184 – 301 mg/kg, which is similar to soya bean meal and fishmeal (National Research Council, 1998; Haasbroek, 2016; van Aswegen, 2019). The 10%CC treatment group had the lowest MCV level, but the 15% CC group had the highest MCV level, cancelling out iron concentration or bioavailability of iron in CC larvae meal as the sole cause for this phenomenon. Keeping in mind that the 10% CC inclusion resulted in the lowest MCV and 15% CC inclusion resulted in the highest MCV, and that diets have a strong influence on MCV (Hackbarth, 1983), slight differences in the diet composition, rather than the dietary inclusion of CC, may have been responsible for the observed difference.

The mean cell haemoglobin concentration (MCHC) is an indication of the amount of haemoglobin relative to the size of the blood cell. Coupled with the low MCV in the 10%CC group is a high MCHC value; an indication that the amount of haemoglobin in the cell did not decrease together with the size of the erythrocyte. This is confirmed by the similar MCH (amount of haemoglobin per red blood cell) and haemoglobin values between treatment groups. Taufek *et al*. (2018) also reported an increase in MCHC levels when fishmeal was replaced with cricket meal in the diets of African catfish, but MCV levels were unchanged in their study. Not only was the MCV for 10% CC chickens in the present study low, the MCV and PCV values for all the treatment groups fell below the normal range for chickens. Considering the blood loss during blood collection the previous weeks, these low values can be expected.

Moreover, PCV is also an index of toxicity in the blood, with high levels suggesting the presence of toxic factors (Pikula *et al*., 2010; Akinola & Etuk, 2015; Arika *et al*., 2016). Therefore, due to similar PCV values between treatments, a presumption can be made that the larvae meal diets did not contain toxic factors different from that in the control diets. As there were no differences for any of the haematological traits, except for MCV and MCHC between some treatment groups, haematological results in this present trial suggests that BSF or CC larvae meal had no negative effects on the health status of the animals.

4.4.5 Organ weight and gizzard erosion

Certain insects can contain toxins that may either be synthesised or accumulated from their substrate (Berenbaum, 1993). Even though Purschke *et al*. (2017) demonstrated that challenging larvae with mycotoxins and pesticides do not result in an accumulation of external mycotoxins or pesticide contaminants in larvae tissue, low levels of naturally occurring mycotoxins have been found on *Chrysomya* spp, *H. illucens* and *M. domestica* larvae (Charlton 2015). A change in organ parameters in animals can be an indication of feed contamination (D'Mello *et al*., 1999), whereas a change in relative lymphoid organ weights in an environmentally controlled trial can be an indication of immunocompetence as well as host exposure to toxins (Grasman, 2002). It was therefore important to observe organ parameters to determine if the larvae species used in the current trial were safe. Results of this trial showed no significant differences for relative organ weight or gastrointestinal tract pH between the CON group and treatments receiving insect meal. Therefore, when only taking these parameters in consideration, CC meal, containing larvae reared on animal offal, did not pose a threat to broilers.

Another natural toxin occurring in insects is histamine (Nässel, 1999; Chomchai & Chomchai, 2018). Not only does small levels of histamine occur in the brain of insects (Nässel, 1999), but insects are high in histidine, which can be decarboxylated by bacteria to histamine, a heat-stable toxin (Chomchai & Chomchai, 2018). Histamine is a major contributor to gizzard erosion in broilers. Overheating of histamine during the processing of raw materials can cause it to react with lysine to form gizzerosine, a toxin which in turn promotes gastric acid secretion when ingested by chickens, causing gizzard erosion (Okazaki *et al*., 1983). Even though small amounts of histamine naturally occurs in insects (Nässel, 1999; Chomchai & Chomchai, 2018), no gizzard erosion was observed in broilers in this study. Similarly, Hall *et al*., (2018) reported an average low gizzard erosion score of 1.56 when up to 60% *M. domestica* was included in broiler diets. Pretorius (2011) evaluated the effect of different drying temperature of M. domestica larvae on gizzard erosion. Even though a few more cases of mild gizzard erosion were recorded in meal dried at 85°C compared to 65°C, there were no significant differences between treatments. It should be noted that storage conditions of insects before processing is important. If insects are not directly heated in an oven after being killed, it should be refrigerated to keep bacterial overgrowth to a minimum in order to prevent bacterial decarboxylation of histidine to histamine, which can cause poisoning in humans and animals (Chomchai & Chomchai, 2018)

4.5 Conclusion

Considering the organ data and immune parameters, the use of CC and BSF larvae meals in broiler diets appears to be safe for chickens. Larvae meal has shown to be a good protein source to partially replace fishmeal and soya bean meal, as no differences were observed for weight gain at slaughter ages of 35 of 42 days; however, BSF meal inclusion levels higher than 10% could compromise FCR.

Due to the enhancement of the cellular and humoral immune responses in larvae fed broilers, it is possible that an improvement in production parameters may be observed when challenged with pathogens. Therefore, challenge studies are recommended to explore in-depth advantages of CC and BSF larvae meal.

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Chapter 5

Inclusion of *Hermetia illucens* larvae reared on fish offal to the diet of broiler quails: effect on immunity and cecal microbial populations

Abstract

Hermetia illucens (black soldier fly; BSF) larvae meal has shown to be a good protein source in monogastric animal diets but published data regarding its immunomodulatory properties in poultry is limited. This trial determined the effects of BSF larvae meal reared on two different substrates (100% commercial layer chicken mash (BSF-M) or 50% commercial layer chicken mash + 50% fish offal (BSF-F)) on certain immune parameters and on selected bacterial counts in the ceca of broiler quails. A total of 300 birds (100 birds/treatment) for production purposes, 60 birds (20 birds/treatment) for immunological purposes and 30 birds (10 birds/treatment) for cecal bacteria counts were randomly allocated to one of three treatments diets: a commercial Japanese broiler quail diet as control (CON) or a larvae meal diet containing either 10% BSF-M or BSF-F larvae meal. Fish offal was chosen to form part of the substrate for larvae in order to increase the content of long-chain omega-3 (n-3) polyunsaturated fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), both known for their immunomodulatory properties. Blood was collected 27 and 37 days after the trial commenced to determine lysozyme concentrations, bactericidal activity, protein fractions of the serum and humoral immune response (antibody titres) to porcine red blood cells. Quails in the BSF-F group exhibited lower slaughter weight compared to the CON and BSF-M fed quails. Quails in the BSF-M group had a significantly higher secondary humoral immune response compared to the CON group. Dietary inclusion of larvae meal significantly increased lymphoproliferative response, with the BSF-F group exhibiting the greatest response. Dietary treatments had no effect on serum bactericidal activity. The majority of serum protein fractions were not influenced by treatment, with the exception of α2-globulin and γ-globulin. For both sampling dates, α2-globulin was significantly higher in the BSF-M and BSF-F groups compared to the CON group. The γ-globulin levels for quails in the BSF-F treatment group were significantly lower than the BSF-M and CON treatment group for the first sampling date, but levels increased after ten days, resulting in concentrations being comparable between treatments. Lastly, feeding the dietary treatments for 20 days had no significant effects on the cecal bacterial counts. To conclude, dietary inclusion of BSF larvae meal has immunostimulatory effects in broiler quails; however, the substrate used to rear the larvae on plays a role in the outcome.

Keywords: Insect meal, maggot meal, humoral immunity, cellular immunity, lysozyme, serum protein, serum bactericidal properties

5.1 Introduction

The increase in costs of soya bean meal and fish meal due to the increase of global food and feed demand has resulted in the exploration of new, sustainable protein sources for the monogastric animal feed industry. With the goal to reduce the impact that animal protein production has on the environment, the use of fly larvae meal as an alternative protein source in animal feed has gained attention in recent years. The majority of studies proved larvae meal to be a good substitute for the conventional protein sources in monogastric diets, resulting in production parameters similar to that of animals fed fish meal and soya bean meal (Pretorius, 2011; Uushona, 2015; Driemeyer, 2016; Maurer *et al.*, 2016; Cullere *et al.*, 2016). Even though a vast amount of research has been done on insect meal as a protein source, its potential health benefits in quails are still unclear.

The fat content of black soldier fly (BSF) meal is usually high, but extremely variable. The fat content of insects is mostly dependent on their rearing substrate and stage of development (Stanley-Samuelson & Dadd, 1983; Jucker *et al.*, 2017; Liland *et al.*, 2017; Meneguz *et al.*, 2018). Many insects have natural occurring long-chain unsaturated fatty acids in their tissue, but the unsaturated fatty acids levels in BSF larvae meal is low compared to crickets, mealworm and house fly larvae meal (Makkar *et al.*, 2014). However, the fatty acid composition of BSF larvae can be manipulated through their diet. For example, St-Hilaire *et al.* (2007) and Barroso *et al.* (2017) managed to favourably increase the omega-3 (n-3) concentration in BSF larvae by adding fish offal or fishmeal to their substrate.

Dietary n-3 polyunsaturated fatty acids (PUFAs) have proven to exhibit health benefits in both human and animals (He *et al.*, 2007; Turchini *et al.*, 2012). Dietary fatty acids and the balance of particular fatty acids, especially the ratio between n-3 and n-6 PUFAs, possess strong immunomodulatory capabilities (Cherian, 2007; Calder, 2011). Dietary PUFAs are important building blocks for cell membrane synthesis, and the cell membrane lipids in return provide the substrate for the synthesis of the communication molecules involved in the immune system. Therefore, dietary PUFAs indirectly determines the type of immune response that follows cell damage (Klasing, 1998). Fish oils rich in n-3 PUFAs enhanced antibody production when added to diets of mice (Prickett *et al.*, 1982) and chickens (Fritsche *et al.,* 1991). In addition, increased n-6:n-3 could decrease mammalian T-lymphocyte proliferation and Natural killer cell activity (Baker *et al.*, 1981; Brunda *et al.,* 1980).

Commercial poultry is vulnerable to stress-related immunosuppression, resulting in vaccination failure, morbidity, and possible mortality in flocks. Therefore, the poultry industry could benefit greatly from the use of dietary immunostimulants. Thus, if larvae meal still possesses the antibacterial, antiviral and immunomodulatory abilities exhibited in larvae extracts (Meylaers *et al.*, 2004; Chu *et al.*, 2011; Yong Wang *et al.*, 2012; Park *et al.*, 2015; Harlystiarini *et al.*, 2019; Auza *et al.*, 2020; Mouithys-Mickalad *et al.*, 2020), and elevated n-3 PUFA concentration in larvae meal exhibit immunostimulatory effects comparable to reported results regarding n-3 levels in animal diets, feeding BSF larvae meal or n-3 enriched BSF larvae meal to monogastric animals may alleviate the need for antibiotics. On the basis of the above-mentioned considerations, the aims of the study were to determine the effects of larvae meal from larvae reared on two different substrates (fish offal or chicken feed) on the growth parameters, selected immune parameters and caecal bacteria counts of broiler quails.

5.2 Materials and methods

5.2.1 Insect rearing

Black soldier fly larvae used in the trial were reared on two different substrates as described in Chapter 3. Briefly, to produce the BSF-M larvae meal, freshly hatched BSF neonatal larvae were reared on a commercial chicken layer mash (CP = 130 g/kg; fat = 25 g/kg; fibre = 70 g/kg; moisture = 120 g/kg; Ca = 35 g/kg; P = 5g/kg; lysine 5g/kg), soaked in hot water (water/mash = 1.6:1) for 16 hours. For the BSF-F larvae meal, a substrate consisting out of 50% soaked layer mash and 50% fish offal (provided by I&J commercial fishing company (Cape Town, South Africa)) were fed to the larvae. Larvae were reared in an environmentally controlled room (temperature of 27 ± 1°C and relative humidity of 65 ± 5%) and harvested after 16 days, before reaching 6th instar (pre-pupae). In order to kill the larvae and inhibit tyrosinase activation and autolysis, larvae were placed in boiling water for one minute. Subsequently, larvae were rinsed to remove all debris, dried in a ventilated drying oven for 16 hours at 65°C and finely ground afterwards. Nutrient composition of the larvae meal sources is provided in Chapter 3

5.2.2 Animals and diets

To determine the immunomodulatory and antimicrobial effects of the dietary treatments, a total of 390 as-hatched ten-day-old Japanese broiler quails were randomly selected from a private quail farm in the Vicenza province (Italy). At the start of the trial, birds were labelled with a wing tag, individually weighed and housed in battery cages in an environmentally controlled room on the farm. The immunological trial ran in conjunction with the performance trial. A total of 300 birds (100 birds/treatment) for growth performance purposes, 60 birds (20 birds/treatment) for immunological purposes and 30 birds (ten birds/treatment) for cecal bacterial counts were randomly allocated to one of three dietary treatments (Table 1). The control diet (CON) was a standard grower diet formulated according to the nutrient requirements for broiler quails. To formulate the BSF-M and BSF-F treatments diets, either 10% BSF-M larvae meal or 10% BSF-F larvae meal was added to the CON diets through partly replacing conventional protein/fat sources while maintaining an iso-nitrogenous and iso-caloric diet. Birds were supplied with *ad libitum* feed and water. At 29 days of age, quails reared for production and slaughter purposes were individually weighed, and feed consumption was recorded on a cage basis (20 birds per pen; n=5). Individual feed intake and feed conversion ratio (FCR) were calculated as an average of the cage after correcting for mortality. The trial protocol was approved by the veterinary authority and carried out according to the article ‘2, DL 4 March 2014, No. 26’ of the Official Journal of the Italian Republic (http://www.gazzettaufficiale.it/eli/id/2014/03/14/14G00036/sg), implementing the EC Directive 86/60963/2010 EU regarding the protection of animals used for experimental and other scientific purposes.

Table 5.1 Ingredient composition, nutrient composition and analysed nutritionally important PUFAs (% of total fatty acids) of the experimental diets

	Treatment diets[1]		
	CON	BSF-M	BSF-F
Ingredients (g/kg)			
Maize (fine)	435.6	450.5	434.8
Soybean meal	460.4	377.0	373.5
Dried *Hermetia illucens* larvae (BSF)	0.0	100.0	100.0
Whole wheat (fine)	23.5	42.5	61.7
Calcium carbonate	21.5	20.0	20.0
NaCl	2.7	2.7	2.7
L-Lysine	0.5	0.5	0.5
DL-Methionine	1.8	1.8	1.8
Vitamin-mineral premix	5.0	5.0	5.0
Soybean oil	49.0	0.0	0.0
Nutritional value (analysed)			
Gross Energy (MJ/kg)	17.4	17.9	17.8
Crude protein (g/kg)	243.3	238.9	239.8
Crude fat	59.8	56.9	55.9
Starch	282.1	314.4	312.6
Ash	63.2	71.8	65.3
Nutritionally important PUFAs (analysed %) [2]			
C18:2 *n*-6 (LA)	51.3	25.1	18.8
C18:3 *n*-3 (ALA)	4.37	1.16	1.35
C20:4 *n*-6 (ARA)	0.06	0.06	0.09
C20:5 *n*-3 (EPA)	0.08	0.05	0.58
C22:6 *n*-3 (DHA)	0.00	0.00	0.10

[1]CON = control diet; BSF-M and BSF-F = control diet supplemented with dried *H. illucens* larvae reared on layer mash (BSF-M) or on 50:50 layer mash and fish offal (BSF-F)
[2]LA= Linoleic acid; ALA= α-Linolenic acid; ARA= Arachidonic acid, EPA= eicosapentaenoic acid; DHA= docosahexaenoic acid

5.2.3 Immunisation with pig red blood cells (PRBC)

On day 20 and 30 of feeding the treatment diets, quails were injected intramuscularly with 0.25 ml of 10% porcine red blood cells (Innovative Research Inc., Southfield, M, USA). Japanese quails respond poorly to immunisation with sheep erythrocytes. Therefore, porcine erythrocytes, which evoke a higher antibody titer in quails (Jackovitz *et al.*, 2016) were used as a T-dependent antigen to quantify the antibody response. Blood was collected from the brachial vein seven days after each injection to determine the primary and secondary antibody response. Blood was transferred into tubes containing clot activator. Serum was separated by centrifugation at 1800 g for ten minutes at 4°C and stored at -20°C until further analysis.

5.2.4 Wing web thickness response to PHA-P (lymphoproliferative response)

The lymphoproliferative response to PHA-P, an indicator of T-cell mediated immune responsiveness in animals, was assessed 20 days after the commencement of treatment diets using the procedures of Corrier & DeLoach (1990). Briefly, a dose of 0.1 ml of 1 mg PHA-P (L8754, Sigma Aldrich, St. Louis, MO, USA)

dissolved in 100 µl phosphate-buffered saline (PBS) was injected intra-dermally into the left-wing web of the birds while the right-wing web received a control injection of 0.1 ml sterile PBS alone. The thickness of each wing web was measured immediately before and 24 hours post-injection with a thickness meter with an accuracy of 0.01 mm. The wing web swelling reactions to PHA-P were calculated using the swelling index calculated with calculation 5.1

Calculation 5.1:

$$\text{Index} = (\text{mm post PHA injection} - \text{mm pre PHA injection}) - (\text{mm post PBS injection} - \text{mm pre PBS injection})$$

5.2.5 Haemagglutination assay

Serum samples used for the haemagglutination assay were placed in a water bath for 30 minutes at 56°C to inactivate the compliment. A volume of 50 µl PBS was added to each well of a 96 well U-shaped bottom microplate. Subsequently, 50 µl of serum was added to the first well of each row, and serial two-fold dilutions were made of each sample into the remaining 11 wells of the row. Fifty µl of 1% PRBC suspension was added to each well. Total antibody titres (HA titre) were read after 30 minutes incubation at 37°C. Titers were expressed as Log_2 of the reciprocal of the highest dilution giving visible agglutination and measured the activity of total (IgM and IgG) haemagglutinating antibodies.

5.2.6 Serum lysozyme concentration

Lysozyme concentration was determined according to the method of Osserman & Lawlor (1966), modified by Bonizzi *et al*. (1989), by means of measuring the lysed areas around the serum samples placed in wells in agar containing *Micrococcus lysodeikticus*. To prepare the plates, 1 g agarose (type II medium EEO; Sigma A-6877) was added to 100 ml sodium phosphate buffer (pH6.3: 7.650 g NaCl + 0.724 g Na_2HPO_4 $_2H_2O$ + 0.210 g KH_2PO_4+ 1000 ml H_2O) and boiled in a water bath until a homogenous solution was obtained. The agar solution was maintained at a temperature of 60°C in a warm bath. A bacterial suspension was prepared by adding 0.1 g *M. lysodeikticus* (M-3770, Sigma-Aldrich, 3770) to 2 ml sodium phosphate buffer. Subsequently 200 µl bacterial suspension was added to each 100 ml agar solution. The homogenous bacterial agar solution (20 ml) was poured into 10 cm square petri dishes and wells of 2 mm diameter, spaced 2 cm from each other were made in the agar with a thin-walled brass tube connected to a vacuum pump. Wells were filled with 20 µl serum (in duplicate). A standard dilution (512, 256 128, 64, 32, 16, 8, 4, 2, 1, 0.5 µg/ml) of purified egg white lysozyme (Sigma-Aldrich, 62971) in saline phosphate buffer (pH 6.3) were run with the test sample. The plates were incubated in a humidified box at 37°C for 16 h. The diameter of the lysed area around the wells filled with serum or lysozyme standards were measured with a calliper. The lysozyme concentration (µg/mL) of the serum samples were proportional to the diameter of lysed areas and were determined from a semi-logarithmic curve created from the purified lysozyme standards.

5.2.7 Serum bactericidal activity

To determine the bactericidal activity of serum, a turbidimetric assay was performed in a 96 well round bottom plate using the method previously described in Amadori *et al*. (1997). Briefly, a non-pathogenic strain of *Escherichia coli* was suspended in 15 ml Brain Heart Infusion broth (BHI), incubated at 37°C until the optical density at 590 nm doubled, an indication that log phase of growth was reached. Sterile saline solution was used to dilute the bacteria to a 1:100 dilution. Subsequently, the 50 µl of serum (in duplicate) were distributed in the wells, followed by 50 µl veronal buffer, 100 µl of BHI broth and 10 µl of the diluted bacterial suspension. A positive control was set up without serum to determine bacterial growth, and a negative control was set up without bacteria to determine the sterility of the components. The missing components in both controls were substituted with veronal buffer at the same volumes. Plates were covered and incubated in a humidified box at 37°C for 18 hours. The microtiter plates were read spectrophotometrically in an ELISA reader at 690 nm with the negative control set as blank. Serum bactericidal activity (SBA) was derived by equation 5.2:

Equation 5.2

%SBA= (OD growth control – OD test sample) / (OD growth control x 100)

5.2.8 Serum protein fractions

Serum biochemistry was determined by means of a Hitachi 912 automatic analyser (Roche Diagnostics GmbH, D-68298 Mannheim, Germany). Serum protein separations were made using the P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Inc., 4300 N. Harbor Blvd., Fullerton, CA, 92835, USA)

5.2.9 Bacterial enumeration from cecal digesta

After receiving the dietary treatment for 20 days, 30 birds (n=10) were killed by means of cervical dislocation. Subsequently, carcasses were opened, the ceca were removed aseptically, and the content of the ceca was placed in a sterile test tube, weighed, and diluted 1:10 with sterile saline solution. Digesta homogenate was serially diluted from 10^{-1} to 10^{-5}. Dilutions were plated in duplicate on selective agar media for enumeration of target bacteria groups. In particular, total viable counts, total coliforms, *Staphylococcus*, *Micrococcus*, *Lactobacillus* spp. and *Aeromonas* + *Pseudomonas* were enumerated using Plate Count agar (CPA), MacConkey agar (MCC), Baird-Parker agar (BP), Mannitol salt agar (MSA), De Man, Rogosa & Sharpe *agar (*MRS) and Glutamate Starch Phenol Red *Agar* (GSP), respectively. Plates were incubated for at 37°C for 24 hours, and colonies were counted.

5.2.10 Statistical analysis

All the parameters were subjected to a one-way ANOVA with experimental diets as a fixed effect, following the GLM procedure of the SAS 9.1.3 statistical analysis software for Windows (SAS Institute, 2008). Differences were considered significant when $P < 0.05$. When significant differences occurred, treatment mean differences were identified by pairwise comparison using the Bonferroni test.

5.3 Results

5.3.1 Production parameters

Dietary inclusion of either larvae meal sources did not affect feed conversion ratio or mortality of broiler quails (Table 5.2). At the end of the trial period, quails in the CON and BSF-M treatment group had significantly higher individual live weight (n=100) and average daily gain compared to birds in the BSF-F group. There was only a tendency (P = 0.052) towards differences for feed intake. It should be noted that due to a lack of available cages, samples size for feed intake (n=5 per diet) were small and most likely contributed to the lack of observed differences between treatments for feed intake.

Table 5.2 Effect of dietary inclusion of *Hermetia illucens* larvae meal on production parameters of Japanese broiler quails (mean ± s.e.)

	Treatment diets[1]			*P*-value
	CON	BSF-M	BSF-F	
Live weight (g)				
Initial weight (10 days)	78 ± 0.73	78 ± 0.39	78 ± 0.43	0.978
Slaughter weight (29 days)	241[a] ± 2.45	248[a] ± 2.36	229[b] ± 2.74	<0.001
Average daily gain (g/day)	9.0[a] ± 0.17	9.4[a] ± 0.16	8.4[b] ± 0.15	<0.001
Average daily feed intake/bird (g)	29.1 ± 0.70	29.9 ± 0.54	27.5 ± 0.54	0.052
Feed conversion ratio	3.4 ± 0.25	3.4 ± 0.13	3.5 ± 0.19	0.843
Mortality (%)	1.0 ± 0.20	1.0 ± 0.20	2.0 ± 0.4	0.638

[1] CON = control diet; BSF-M and BSF-F = control diet supplemented with dried *H. illucens* larvae reared on layer mash (BSF-M) or on 50:50 layer mash and fish offal (BSF-F)
[a,b,c] Means within rows with different superscripts differ significantly (*P* <0.05)

5.3.2 Humoral antibody response, lysozyme activity, serum bactericidal activity and cellular immune response

A weak primary humoral immune response was observed for all the treatments with no significant differences for antibody production (Table 5.3). After the second immunisation with PRBC, HA titres were significantly higher for the BSF-M (titre = 5.7) treatment group compared to the CON treatment group (titre = 3.5), with titres for the BSF-F (titre = 4.6) treatment group being intermediate. Serum lysozyme activity only significantly differed between treatments on day 37, with higher levels observed in the BSF-F (206 µg/ml) treatment group compared to the CON (91 µg/ml) and BSF-M (125 µg/ml) treatment group. No significant differences were observed for serum bactericidal activity on either of the sampling days. The wing web thickness reaction to PHA-P was significantly influenced by all the treatments, with the greatest response in quails in the BSF-F treatment, followed by BSF-M quails, and lastly with quails in the CON treatment exhibiting the lowest response.

Table 5.3 Effect of dietary inclusion of *Hermetia illucens* larvae meal on the humoral immune response, lysozyme activity, serum bactericidal activity and cellular immune response measured in Japanese quails (mean ± s.e)

	Treatment diets[1]			*P*-value
	CON	BSF-M	BSF-F	
Haemagglutination titre				
Primary antibody response	0.41 ± 0.24	0.82 ± 0.29	0.77 ± 0.41	0.180
Secondary antibody response	3.5^{b} ± 0.50	5.7^{a} ± 0.45	4.6^{ab} ± 0.37	0.006
Lysozyme activity (µg/ml)				
Day 27	150 ± 20	165 ± 24	239 ± 53	0.173
Day 37	91^{b} ± 9	125^{b} ± 20	206^{a} ± 23	<0.001
Serum bactericidal activity (%)				
Day 27	68 ± 1.45	62 ± 3.09	58 ± 4.33	0.160
Day 37	51 ± 3.84	49 ± 5.80	51 ± 3.61	0.954
Wing web swelling index (mm)	0.27^{c} ± 0.05	0.66^{b} ± 0.09	1.03^{a} ± 0.07	<0.001

[1] CON = control diet; BSF-M and BSF-F = control diet supplemented with dried *H. illucens* larvae reared on layer mash (BSF-M) or on 50:50 layer mash and fish offal (BSF-F)
[a,b,c] Means within rows with different superscripts differ significantly (P <0.05)

5.3.3 Serum protein fractions

For serum collected on day 27, no significant differences were observed for total protein, pre-albumin, albumin, α1-globulin, or β1-globulin concentrations (Table 5.4), however dietary inclusion of BSF-M or BSF-F larvae meal significantly increased α2-globulin concentration compared to the CON treatment (8.02 g/L, 7.57 g/L vs 6.11 g/L, respectively). Dietary inclusion of HSF-F meal significantly decreased γ-globulin compared to the BSF-M and CON treatment groups on day 27. Serum protein and protein fractions were overall comparable on day 37. The only exception was α2-globulin, being higher in the two larvae meal treatment groups when compared to the CON treatment (8.62 g/L; 8.38 g/L vs 7.26 g/L, respectively).

5.3.4 Cecal bacterial counts

No differences were noticed between treatments for the microbial composition of the cecal content when considering total viable counts, total coliforms, *Staphylococcus*, *Micrococcus*, *Aeromonas*, *Pseudomonas* or *Lactobacillus* spp. (Table 5.5).

Table 5.4 Effect of dietary inclusion of *Hermetia illucens* larvae meal on serum protein (g/L) and serum protein fractions (g/L) measured in Japanese quails (mean ± s.e)

	Treatment diets[1]			*P*-value
	CON	BSFM	SF-F	
Day 27				
Total protein	27.42 ± 0.70	30.21 ± 4.40	27.67 ± 1.22	0.22
Prealbumin	0.45 ± 0.16	0.28 ± 0.04	0.31 ± 0.04	0.25
Albumin	11.00 ± 0.72	12.24 ± 0.92	11.85 ± 3.11	0.56
α1-globulin	0.83 ± 0.11	0.70 ± 0.08	0.86 ± 0.67	0.53
α2-globulin	6.11[b] ± 0.22	8.02[a] ± 0.27	7.57[a] ± 0.25	0.01
β1-globulin	6.21 ± 0.59	6.04 ± 0.50	5.10 ± 0.44	0.24
γ-globulin	2.84[a] ± 0.39	2.91[a] ± 0.29	1.97[b] ± 0.21	0.04
Albumin/Globulin	0.69 ± 0.04	0.69 ± 0.04	0.75 ± 0.03	0.34
Day 37				
Total Protein	31.46 ± 1.76	31.00 ± 2.01	30.69 ± 1.52	0.96
Prealbumin	0.62 ± 0.18	0.41 ± 0.12	0.36 ± 0.06	0.35
Albumin	12.35 ± 0.95	11.31 ± 0.92	10.44 ± 0.76	0.37
α1-globulin	0.86 ± 0.66	0.66 ± 0.48	0.68 ± 0.48	0.28
α2-globulin	7.26[b] ± 0.26	8.62[a] ± 0.36	8.38[a] ± 0.32	0.01
β1-globulin	7.47 ± 0.68	6.81 ± 0.90	7.20 ± 1.10	0.87
γ-globulin	2.50 ± 0.27	3.40 ± 0.35	3.64 ± 0.511	0.14
Albumin/Globulin	0.67 ± 0.04	0.59 ± 0.03	0.54 ± 0.04	0.07

[1] CON = control diet; BSF-M and BSF-F = control diet supplemented with dried *H. illucens* larvae reared on layer mash (BSF-M) or on 50:50 layer mash and fish offal (BSF-F)
[a,b] Means within rows with different superscripts differ significantly (P <0.05)

Table 5.5 Effect of dietary inclusion of *Hermetia illucens* larvae meal on selected microbial count (CFU/g) in the cecal content of Japanese quails (mean ± s.e)

	Treatment diets[1]			*P*-value
	CON	BSF-M	BSF-F	
Total viable count	8.21 ± 0.26	7.30 ± 0.32	7.43 ± 0.36	0.11
Total Coliforms	6.54 ± 0.45	6.05 ± 0.45	6.55 ± 0.46	0.67
Staphylococcus	3.06 ± 0.12	3.02 ± 0.33	2.93 ± 0.21	0.93
Micrococcus	4.29 ± 0.36	4.69 ± 0.36	3.91 ± 0.28	0.20
Aeromonas	2.60 ± 0.46	3.07 ± 0.46	3.31 ± 0.48	0.55
Pseudomonas	6.38 ± 0.16	5.61 ± 0.30	5.70 ± 0.30	0.10
Lactobacillus spp.	3.82 ± 0.12	4.14 ± 0.23	3.85 ± 0.15	0.35

[1] CON = control diet; BSF-M and BSF-F = control diet supplemented with dried *H. illucens* larvae reared on layer mash (BSF-M) or on 50:50 layer mash and fish offal (BSF-F)

5.4 Discussion

5.4.1 Omega-3 enrichment of larvae meal

An objective of this study was to enrich larvae meal with the two specific long-chain n-3 PUFAs, EPA (20:5n-3) and DHA (22:6n-3), also known as marine n-3 fatty acids, considering they are only produced in fish. Adding 10% of larvae meal from larvae raised on 50% fish to the diets of quails, resulted in EPA and DHA levels of 0.58% and 0.10% in the BSF-F diet, respectively. This is a slight increase compared to the levels in the BSF-M (EPA=0.05%; DHA=0.00%) and CON (EPA= 0.08%; DHA=0.00%) diets. It should be noted that even though the total n-3 levels of the CON diet were high, this is only due to the high (more than double) level of α-linolenic acid (ALA, C18:3 n-3), a short-chain n-3 PUFA (Table 1).

5.4.2 Production parameters

Seeing as the nutrient composition of the two diets in terms of protein, crude fat, starch, and gross energy were similar, the lower weight gain of quails in the BSF-F treatment group was unexpected. Aromas of the diet can influence the acceptance of the diet by poultry (Alenier & Combs, 1981). It can be hypothesised that the fish aroma/flavour of the BSF-F diet made it less palatable than the other two diets, as quails in this treatment group exhibited a tendency (P = 0.0529) towards reduced feed intake. It should be noted that the oxidative status of the diets was not determined during the trial, and a different aroma to the BSF-F larvae meal was observed closer to the end of the trials period. Lipid peroxidation in dietary oils gives rise to hydroperoxides, which in turn can be modified into compounds that develop off-flavours in rancid oils (Baker & Davies, 1996). A possibility exists that the high n-3 fatty acids in the BSF-F diet, after exposure to heat and oxygen in the broiler house, could have undergone oxidative deterioration over time (Kaitaranta, 1992). Oxidative changes give rise to unpleasant flavours in diets containing high amounts of DHA and EPA (Kaitaranta, 1992). Since unpleasant flavours and lipid-hydroperoxides in rancid dietary oil will decreases feed intake, a negative effect on growth rate can be expected when animals are fed rancid diets (Baker & Davies,

1996). For this reason, the use of antioxidants in the diets should be considered when larvae meal reared on fish offal is used for quail feed purposes.

5.4.3 Immune parameters

It is well known that the two long-chain n-3 PUFAs (EPA and DHA) exhibit health benefits for humans (Turchini *et al.*, 2012) and immunomodulatory effects in animals (He *et al.*, 2007), but limited evidence exists that indicates health benefits from dietary ALA. Most seeds are high in linoleic acid (LA, C18:2 n-6); however, linseed and soya have considerable amounts of ALA. The CON diet contained 4.9% soya oil, explaining the high ALA content. Linolenic acid can be converted to n-3 long-chain PUFAs, but its conversion efficiency to DHA is very low. Taking humans as an example, the conversion efficiency ranges between 1% for infants to 0% for adults (Brenna *et al.*, 2009). On the other hand, LA is usually converted into arachidonic acid (AA, 20:4n-6). Arachidonic acid and long-chain n-3 PUFAs compete for incorporation into cell membrane phospholipids. Both AA and DHA are precursors of eicosanoids by means of cyclooxygenase and lipoxygenase, but the eicosanoids derived from AA in membranes, including thromboxanes and prostaglandin E_2 (PGE_2), are more pro-inflammatory than those derived from DHA (Hwang, 1989; Calder, 2006). Prostaglandin E_2 is also well known for its immunosuppressive effects (Hwang, 1989). Therefore the levels of DHA or AA deposited in the cells, as a result of the long-chain n-3:n-6, will have considerable effects on the function of the immune system. Taking into account the high LA levels (precursor of AA) and 0% DHA in the CON and BSF-M diets, the possibility exists that the low concentrations of EPA and DHA in the BSF-F diet could still alter certain immune parameters.

Acquired immunity has two arms, namely humoral immunity and cellular immunity. The humoral immunity is mediated by serum antibodies, secreted by B-cells after binding with antigens. The quails in this study were challenged twice with PRBC, a non-pathogenic antigen, to evoke a primary and secondary humoral immune response, measured by a haemagglutination assay. A relatively small primary humoral response was detected, with no differences between the treatment groups. However, after the second PRBC immunisation, the secondary response of the BSF-M group was significantly higher compared to the CON treatment group, with the BSF-F group being intermediate. Publications regarding the effect of larvae meal on the humoral immune response in animals are scarce. Driemeyer (2016) reported dietary BSF larvae meal to have no significant effect on the normal antibody concentrations in weaner piglets, then again, the piglets were not immunised with any known antigens during their trial. Similarly, dietary mealworms had no effect on general immunoglobulin concentrations in uninfected broilers (Jin *et al.*, 2016). Neither did the antibody response increase after the vaccination of salmon receiving BSF larvae meal (Li *et al.*, 2019). According to our knowledge, the current study is the first to determine antibody production ability of immunized poultry receiving dietary larvae meal. Despite the HA titre for the BSF-F group not differing significantly from the BSF-M groups, the antibody production was not as large as in BSF-M quails, owing to a HA titre similar to the CON group. The reason for the slight differences in response in the two larvae meal treatment groups is unclear. Previous studies that examined the effect of n-3:n-6 on the humoral immune response by adding fish oil to poultry diets were either unsuccessful in changing the HA titre, or positive results were observed (Fritsche *et al.*, 1991; Guo *et al.*, 2004; Khatibjoo *et al.*, 2011).

At day 27 of the trial, no differences in serum lysozyme activities were observed among treatments. However, on day 37, quails in the BSF-F treatment group had significantly higher lysozyme activity compared to chicks receiving the CON or BSF-M diet. Lysozymes are glycoside hydrolases secreted by phagocytes and are involved in the innate immune response of animals; exerting a synergic action with the compliment system and the humoral immune response (Wardlaw, 1961). Lysozymes within these phagocytes hydrolyse the mucopeptide in bacterial cell walls, resulting in lysis of some Gram-positive bacteria (Chassy & Giuffrida, 1980). Furthermore, certain antibodies such as IgA are only effective in lysing *E. coli* in the presence of both lysozyme and complement (Adinolfi *et al.*, 1966). Higher levels of lysozyme activity in sera of the BSF-F could be an indication that larvae meal reared on fish has certain properties to stimulate the activation of these phagocytes, enhancing the antibacterial defence mechanisms of quails. The mechanism might be the decreased production of certain metabolites such as PGE_2 due to the increased long-chain n-3 PUFAs and the shift in n-3/n-6, which in turn transforms the membrane composition of immune cells (Calder, 2007). A negative correlation exists between the level of long-chain n-3 in the diet and the production of PGE_2 (Guo *et al.*, 2004), whereas n-6 fatty acids enhance the synthesis of PGE_2 of peripheral blood leucocytes (Konieczka *et al.*, 2007). More importantly, PGE_2 negatively correlates with lysozyme activity (Guo *et al.*, 2004; He *et al.*, 2007). Furthermore, in the current study, DHA was only detected in the BSF-F feed. Protectin D1, a mediator from DHA, enhances the phagocytic activity of macrophages (Schwab *et al.*, 2007), the primary producer of lysozyme in the blood (Hansen & Karle, 1977; Venge, 2016); hence partly explaining the increased lysozyme concentration in serum from quails in the BSF-F treatment group. Even though no similar studies could be found on the effect of larvae meal or n-3 enriched larvae meal on serum lysozyme activity in poultry, previous studies indicated an enhancement in serum lysozyme when animals received n-3 enriched diets (Guo *et al.*, 2004; He *et al.*, 2007).

As mentioned above, lysozymes exert a synergistic action with the compliment, a system composed of serum proteins that react against pathogens through a molecular cascade, resulting in microbial lysis. Lysozyme, together with the compliment and antimicrobial peptides (AMP's) in blood, are all responsible for the bactericidal properties serum holds (Riera Romo *et al.*, 2016). It should be noted; even though dietary treatments in this study resulted in differences in serum lysozyme activity against Gram-positive bacteria, no differences were observed when taking into account the total serum bactericidal activity against the Gram-negative bacteria, *E. coli.*

In the present study, the inclusion of insect meal in the diets of quails enhanced cellular immune responses when considering a swelling response to PHA-P. An induced swelling of the wing web 24 hours after mitogen injection can be an indication of an enhanced lymphoproliferative ability of the immune system. Phytohemagglutinin (PHA-P) is a T-cell mitogen used to measure T-lymphocyte function. Injecting PHA-P into the skin of animals stimulates T-cell proliferation, differentiation, and cytokine production, causing an influx of basophils and other leucocytes into the injection site, resulting in swelling of the skin (Stadecker *et al.*, 1977; Corrier & DeLoach, 1990; Grasman, 2002). In addition, results in this study demonstrated that cellular immune responses could be even further enhanced when larvae meal is enriched with long-chain n-3 fatty acids. These results are in agreement with others (Korver & Klasing, 1997; Weng, 2002; Agazzi *et al.*, 2004) who showed that the inclusion of n-3 in animal diets by means of fish oil, increases the swelling response. Granted that PHA-P stimulates T-cell proliferation and PGE_2 suppresses it (Baker *et al.*, 1981), this result can be expected.

Contrary to these observations, Khatibjoo *et al.* (2011) reported a smaller swelling response in animals when dietary n-3:n-6 ratios were low and hypothesized it to be due to the reduced levels of AA and pro-inflammatory AA-derived eicosanoids deposited into the cells. However, as reported by Korver & Klasing (1997), the inclusion of fish oil in the diets of broilers improves the PHA-P response, but decrease indices of inflammatory response. Moreover, Konieczka *et al.* (2007) demonstrated that even though the levels of pro-inflammatory eicosanoids (PGE_2 and thromboxane), decreased in the blood of n-3 enriched chickens, basophil and macrophage counts still increased after PHA-P injection.

Serum proteins have various physiological roles and are a significant indicator of the bird's health condition, including immune and inflammation responses. Total serum protein for quails receiving the three dietary treatments was similar with average levels for treatments being 27.4, 30.21 and 27.67 g/L for the CON, BSF-M and MSF-F treatment groups, respectively. Total serum protein is mainly used for diagnostic tests, with an average concentration of 30-50 g/L for most avian species. A reduction of total serum protein can be an indicator of malnutrition, malabsorption, heavy metal poisoning, intestinal parasitism, prolonged stressed or liver poisoning. However, in spite of the slight decrease in average serum protein levels in CON and BSF-F quails resulting in levels lower than the average for avian species, there were no significant differences between the treatments. The low total protein levels are therefore not treatment-related but rather due to other environmental factors.

Albumin and the four globulin fractions (α1, α2, β, and γ-globulins) are five of the main fractions of serum protein. Many acute-phase proteins can be classified under α-globulins. In the current trial, α2-globulin levels were significantly higher in the BSF-M and BSF-F treatment compared to the control treatment for both sampling days. Although an increase in α-globulins or β-globulins can be an indication of inflammation, certain proteins such as α2-macroglobulin, haptoglobin and ceruloplasmin, classified under α2-globulin, can hold numerous benefits for the animal. Not only does ceruloplasmin scavenge free radicals (Cray 2009), but haptoglobin, or its avian counterpart called PIT54, binds to free haemoglobin, preventing oxidative damage and loss of iron. A reduction of free iron limits the availability of iron needed for bacterial growth. For this reason, haptoglobin is believed to exhibit bacteriostatic properties (Eaton *et al.*, 1982). Lastly, α2-macroglobulin and homologues of this macroglobulin detected in poultry (Starkey & Barrett, 1982; Péault, 1987; Jaarsveld *et al.*, 1994) inactivates toxins (Borth, 1992), removes enzymes released during injury (Cray *et al.*, 2009) and is a protease inhibitor capable of inhibiting both endogenous and exogenous proteases (Magor, 2001). Proteases secreted by parasites serve an important role in parasitic virulence; however, α2-macroglobulin can bind these proteases, resulting in their degradation by means of lysosomes (Van Leuven *et al.*, 1978). It should be noted that all the quails in this study appeared healthy, therefore, despite the fact that α-globulins can be associated with inflammation and considering the beneficial effects of the α2-globulin proteins, the increased α2-globulin levels in the serum of BSF-M and BSF-F quails can be an indication that BSF larvae meal holds immunostimulatory properties in this regard.

The γ-globulin fraction represents the immunoglobulins (antibodies) in the serum and considers the normal immunoglobulin levels together with the increased levels after a humoral immune response to an antigen. Even though the HA titre as a response to PRBC did not differ after the first immunisation, the γ-globulin levels of quails in the CON and BSF-M group were significantly higher compared to those in the BSF-F group. However, after the second immunisation, the γ-globulin concentration in BSF-F quails almost doubled

while the response in the CON and BSF-M treatment groups were relatively small, therefore diminishing the differences observed on day 27. Published data on the effect of fly larvae meal on serum protein in poultry is scarce; however, Bovera *et al.*, (2015) and Biasato *et al.* (2017) measured total protein, albumin and total globulin levels in chickens fed mealworms. Similar to the current study, insect meal had no effect on these parameters; however, Bovera *et al.* (2015) noted a smaller albumin/globulin ratio in chickens fed insect meal and attributed the improved immune response to the properties to the chitin content of insects.

5.4.4 Cecal bacterial counts

Chitin is believed to serve as a prebiotic in the large intestine of poultry (Bovera *et al.,* 2015). However, Khempaka *et al.* (2011) reported that purified chitin was incapable of altering the intestinal populations of *Salmonella*, *E. coli* or *Lactobacillus* in broilers, whereas the chitin constituent in shrimp meal, represented in the form of a chitin-protein complex, resulted in a favourable shift in the intestinal microflora. Hence the fact that chitin also combines with proteins in the cuticles of insects, the favourable shift in intestinal microflora can also be expected in poultry consuming insect meal. However, different from expected, there was no shift in cecal microbial populations when considering the bacteria enumerated in this study. This is consistent with work published by Cullere *et al.* (2016). Although this may be true for the few bacterial populations tested in this study, a different outcome may be expected when taking the entire microbial ecology of the gastrointestinal tract into account. By means of 16S rDNA sequencing, Borrelli *et al.* (2017) showed that BSF larvae meal in the diets of laying hens could cause a major beneficial shift in the cecal microbiota when taking into account microbial species as well as relative abundance. The insect diet increased the numbers of bacteria capable of degrading chitin and increased the richness of the microbiota, which in turn is usually associated with a good health status, whereas a smaller microbial diversity can be the cause of declined immunological and gut protective functions (Sekirov *et al.*, 2010).

5.5 Conclusion

BSF-M meal can be provided to broiler quails at a 10% inclusion rate without negatively affecting production parameters. On the other hand, the negative effect that dietary BSF-F meal had on the growth performance should be further investigated. The focus should be placed on the shelf-life of larvae with increased n-3 levels since this phenomenon could have caused the lowered intake and growth that were recorded in this study. In summary, feeding quails with larvae meal from larvae that were reared on fish offal (with higher levels of n-3 PUFA's), resulted in an improved cell-mediated immunity and an increase in serum lysozyme concentrations. On the other hand, quails receiving larvae meal that were reared on chicken feed had an improved secondary immune response.

Dietary treatments did not alter serum bactericidal activity, cecal bacterial counts or most of the serum protein fractions; however, both larvae meal sources increased α2-globulin concentrations in the serum. It can be concluded that BSF larvae meal has immunostimulatory effects in broiler quails, although the larvae's rearing substrate influences the immune response by the animal.

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Chapter 6

The antimicrobial and immunomodulatory properties of dietary *Chrysomya chloropyga* and *Hermetia illucens* larvae meal in broilers challenged with *Salmonella* Enteritidis

Abstract

Broiler meat can be a source of *Salmonella* contamination for humans. Several factors in fly larvae inhibit *Salmonella* growth *in vitro*, but data regarding its *in vivo* antimicrobial and immunomodulatory properties are lacking. To test the antimicrobial and immunomodulatory efficacy of *Chrysomya chloropyga* (CC) and *Hermetia illucens* (BSF) larvae meal, a challenge experiment with *Salmonella* serotype Enteritidis A9 was conducted. A total of 595 broiler chicks were randomly assigned to five groups given either a control diet (CON+SAL or CON-NEG), a control diet supplemented with oxytetracycline antibiotic (ANTIBIO+SAL), a diet containing 10%CC larvae meal (CC+SAL) or 10% BSF larvae meal (BSF+SAL). Each group consisted out of 17 replicate cages with seven birds/cage. All the chickens, except those in the CON-NEG group, were orally infected with 9×10^7 CFU of *Salmonella enterica* serovar Enteritidis A9 on day nine and ten. One bird per cage was slaughtered on day 11, 14, 21, 24 and 28 for ceca and blood collection. Both larvae meal sources improved live weight in the starter phase, but the effects were diminished by day 28. The feed conversion ratio (FCR) for the CC+SAL, BSF+SAL and ANTIBIO+SAL treatment groups were significantly improved compared to the CON+SAL treatment group. Oxytetracycline significantly reduced *Salmonella* colonisation one- and four-days post-infection, but ceca *Salmonella* levels increased significantly over time, resulting in mean log CFU per ceca of the ANTIBIO+SAL treatment being similar to the CON+SAL group on day 21, 24 and 28. The opposite was noticed for the CC+SAL group, with CFU per ceca counts being significantly lower than the CON+SAL group on day 28. Black soldier fly larvae meal had no significant effect in decreasing *Salmonella* colonisation on either of the slaughtering days. Treatment had no effect on lymphoid organ weight, haematological parameters, or serum interferon-gamma (IFN- γ) levels. *Salmonella* infection significantly increased serum bactericidal activity against *Salmonella* in all the treatment groups one-day post-infection. The inclusion of either larvae meal sources significantly enhanced serum bactericidal activity when compared to the CON+SAL group. Lymphoproliferative response to the PHA-P test was significantly higher in the CC+SAL, BSF+SAL and ANTIBIO+SAL treatment groups. Lastly, the inclusion of BSF larvae meal significantly increased serum lysozyme concentrations after infection, with the CC+SAL group being intermediate to the BSF+SAL and CON+SAL treatment groups. In conclusion, either source of larvae meal can be fed to *Salmonella* infected broilers to enhance immune response, although only long term feeding of CC larvae meal was effective in decreasing *S*. Enteritidis counts in the ceca of broilers.

Keywords: *Hermetia illucens*; *Chrysomya chloropyga*; antibiotics, *Salmonella enterica*; oxytetracycline

6.1 Introduction

Salmonellosis remains a global public health concern. Previous studies have estimated that *Salmonella* causes about 93.8 million cases of gastroenteritis globally each year (Majowicz *et al.*, 2010). Of these reported cases, it was estimated that 80.3 million cases were food-borne (Majowicz *et al.*, 2010). The poultry industry plays a major role in the spreading of *Salmonella*, as previous studies confirmed that a substantial amount of *Salmonella* isolated from South African abattoirs and environmental samples are poultry derived (Magwedere *et al.*, 2015). *Salmonella* prevalence in samples collected from South African poultry farms or store-bought chicken ranged from 19.2% to 51% (Nierop & Duse, 2005; Zishiri, 2016), with the majority being multiple drug-resistant isolates (Zishiri, 2016).

Antibiotic growth promoters such as oxytetracycline were popular for their use in monogastric animals. These compounds possess antimicrobial, immunomodulatory and anti-inflammatory properties, resulting in improved feed conversion ratio's (FCR) (Khadem *et al.*, 2014). The injudicious usage of antibiotics in the poultry industry to control pathogens and act as growth promoters has resulted in the emergence of antibiotic-resistant bacterial strains. The emergence and spread of antibiotic-resistant *Salmonella* in the production systems and in broiler meat have major public health implications. It is therefore important to control the prevalence of *Salmonella* in broilers to minimise the quantity of contaminated meat entering the food chain, which eventually leads to food-borne infections in humans.

Several alternative feed additives, i.e. probiotics (Higgins *et al.*, 2010), fatty acids (Fernández-Rubio *et al.*, 2009) and bacteriophages (Fiorentin *et al.*, 2005) have been proposed to maintain bird gut health and decrease the presence of pathogens such as *Salmonella*. However, if a raw material included in broiler diets contains antibacterial activity against these pathogens or has the ability to increase the innate immune system, the need for additional feed additives might decrease. Insects possess several properties that could play a part in reducing *Salmonella* colonisation in the gastrointestinal tract (GIT) of animals. These properties include high lauric acid content of fly larvae (Spranghers *et al.*, 2018), antimicrobial properties of chitin and it's derivatives (Benhabiles *et al.*, 2012), increased butyrate and acetate production in the chicken ceca due to chitin (Cutrignelli *et al.*, 2018), immunostimulatory properties of protein fractions from larvae (Ai *et al.*, 2013) and production of antimicrobial peptides by larvae (Čeřovský *et al*. 2010; Wang *et al*. 2017)

Hermetia illucens (black soldier fly or BSF) larvae contain high levels of lauric acid which has been documented to exhibit bacteriostatic properties (Lieberman *et al.*, 2006), especially against Gram-positive bacteria (Skřivanová *et al.*, 2005). Furthermore, Hoffman *et al*. (2001) verified the bacteriostatic properties of lauric acid against *Salmonella* Enteritidis. Fat extracted from black soldier fly larvae also effectively reduced proliferation of potentially pathogenic bacteria, *Enterobacteriaceae spp.* in the jejunum of turkeys (Sypniewski *et al.*, 2020). In addition, the exoskeleton of insects contains chitin, which may also contribute to the inhibition of *Salmonella* colonisation. Even though most of the published work focusses on the antimicrobial properties of chitin derivatives like chitosan, Benhabiles *et al*. (2012) observed bacteriostatic properties for both chitin and chitosan. Furthermore, chitin as a feed additive in broiler increases cecal concentrations of butyric acid (Khempaka *et al.*, 2011), which has antimicrobial activity against *Salmonella* (van Immerseel *et al*., 2004; van Immerseel *et al*. 2005). Lastly, peptide extracts from *Musca domestica* (Hou *et al*. 2007), and BSF larvae (Park

et al., 2014; Harlystiarini *et al.*, 2019; Auza *et al.*, 2020) have been shown to inhibit food pathogens such as *S. typhimurium* and *E. coli* by lysing the cytoplasmic membrane (Wang *et al.*, 2010).

Interestingly, 57 peptides from BSF larvae and flies were identified on the gene level, of which a total of 52 were predicted to have antimicrobial activity (Moretta *et al.*, 2020). Extracts from insects not only display antimicrobial properties, but also immunomodulatory properties. For example, Dipterose, a polysaccharide extracted from melon fly pupae, activates the innate immune system against various pathogenic microorganisms by inducing cytokines production by macrophages (Ohta *et al.*, 2016). Likewise, Alloferon, a peptide extracted from blowflies, has been shown to improve important effector mechanisms of the innate immunity by stimulating natural killer lymphocytes *in vitro* and inducing interferon (IFN) production *in vivo* (Chernysh *et al.*, 2002). Moreover, it is well known that cytokines such as interferon-gamma (IFN-γ) are important in the first line of defence against *Salmonella* infection (Lalmanach & Lantier, 1999). Therefore, insect-derived peptides could also indirectly control *Salmonella* levels by modulating the immune system. However, it remains to be elucidated whether BSF and *Chrysomya chloropyga* (CC) larvae synthesise similar analogous peptides with immunostimulatory properties.

Due to the lack of published data on the *in vivo* antibacterial efficiency and immunostimulatory properties of larvae meal in chickens challenged with pathogenic microorganisms, a challenge trial using *Salmonella* Enteritidis A9 was performed on broilers receiving either BSF or CC larvae meal in their diets. An objective of this trial was to evaluate the effects of dietary CC or BSF larvae meal on production parameters (weekly weight gain, feed intake, feed conversion ratio) of *Salmonella* infected broilers; and to compare these parameters with production results from similarly infected broilers supplemented with oxytetracycline, an antibiotic growth promotor. Another objective was to determine if peptide extracts from *C. chloropyga* and BSF larvae meal possessed *in vitro* antimicrobial activity against *Salmonella* and if the use of CC or BSF larvae meal in broiler diets could decrease *Salmonella* colonisation in their ceca over time. The *in vivo Salmonella* reduction efficiency of larvae meal was compared against the activity of oxytetracycline against *S.* Enteritidis A9. The last objective was to determine the immunomodulatory effects of dietary BSF, CC or oxytetracycline on selected immune parameters (lymphoid organ weighs, serum lysozyme concentration, serum bactericidal activity, lymphoproliferative response to a mitogen, haematological parameters, and serum IFN-γ levels) in broilers infected with *Salmonella*.

6.2 Materials and methods

6.2.1 Larvae rearing, drying and nutrient composition

A detailed description of the larvae rearing, drying, and nutrient composition are presented in Chapter 3. Briefly, *C. chloropyga* (CC) larvae were reared on swine offal, consisting out of liver, lungs, kidneys, hearts, and spleens. Larvae were reared on the substrate for approximately five days. Larvae were harvested at pre-pupae stage when migration from the substrate took place. Larvae (250 g) were killed by placing them in 2 L of boiling water for one minute. Larvae were subsequently rinsed three times with water and dried in a ventilated drying oven at 77°C for eight hours (until dry matter content reached 92%). Due to the unavailability of large amounts of kitchen waste, *H. illucens* (BSF) larvae were reared on a formulated diet containing maize,

soya bean oilcake, blood meal, vitamin-mineral premix, lysine and methionine. Larvae were harvested prior to the pre-pupae stage. Larvae (250 g) were killed by placing them in boiling water for one minute. Larvae were then rinsed three times with water and dried in a ventilated drying oven at 77°C for 16 hours (until dry matter content reached 92%). Dried larvae (BSF and CC) were ground into a fine meal (± 2 mm) using a food processor.

6.2.2 Peptide extraction and antimicrobial activity determination

To determine if CC and *H. illucens* larvae produced antimicrobial peptides which are still active after processing, a peptide extraction was performed and tested against *Salmonella* and *E. coli*. To perform the peptide extraction from raw larvae, larvae were killed with liquid nitrogen. For the larvae meal extraction, larvae meal produced for trial purposes were used.

Larvae or larvae meal samples of 5g each (3 replications) were added to 50 ml 70% (V/V) acetonitrile, diluted with Milli-Q water. The raw larvae were homogenised with the acetonitrile solution using a high-speed homogeniser. Samples were kept at room temperature for 24 hours for the extraction to occur. Subsequently, samples were centrifuged for ten minutes at 8600 x g to remove any insoluble material. The supernatant was collected and suspended into a round bottom flask of which the weight was predetermined. The round-bottom-flask containing the supernatant was covered with tissue paper and carefully spun in liquid nitrogen until the supernatant was frozen, covering the sides of the flask. Subsequently, the samples were freeze-dried. After freeze-drying, the mass of the extract was recorded within the flask by subtracting the flask's weight. Samples were made up to 80 mg/ml peptide using 2% ethanol diluted with Milli-Q water. The peptide mass was extracted from the flask by sonicating the extract with the ethanol solution for ten minutes.

A spot plate assay determined growth inhibition of the peptide extract against *E. coli* and *Salmonella*. Extracted peptides were made up into two concentrations of 80 mg/ml and 40 mg/ml. A colony of *E.coli* and *Salmonella* were inoculated in 20 ml Luria-Bertani (LB) media and incubated on a shaker at 37°C. The *Salmonella* and *E. coli* cultures were removed when an OD_{600} of 0.25 and 0.6 (representing the exponential phase of the organisms' growth) was reached when measured in a spectrophotometer. The cells were sub-cultured by inoculating 200 µl of the culture to 20 ml LB media. The sub-culture was incubated until the exponential phase was reached. A total of 700 µl of the subcultures were inoculated on LB agar plates and spread evenly across the surface of the plate by tilting the plate, with any excess culture being poured off the plates. After the plates dried in the laminar flow cabinet, 5 µl of each extract was spotted onto the plates (three replications) and were allowed to dry. For the negative and positive controls on the spot-plate, 2% ethanol solution and oxytetracycline were used, respectively. After 24 hours of incubation at 37ºC, inhibition zones of the peptide extractions were recorded and was noted as: extra-strong inhibition similar to the antibiotic control (++++); strong inhibition (+++); moderate inhibition (++); weak inhibition (+); and no inhibition (--).

6.2.3 Animals and diets

The animal trial was conducted on Mariendahl experimental farm (Stellenbosch, Western Cape, South Africa). Ethical approval to conduct the study was granted by the Research Ethics Committee: Animal Care and Use of Stellenbosch University, Stellenbosch (registration number SU-ACUD16-00043). A total of 595 one-day-old broiler chicks (Ross 308) were obtained from a commercial hatchery. Upon arrival, chicks were sexed, labelled according to sex, weighed, and housed in an environmentally controlled room. Seven chicks (four males and three females) were allocated to each of 85 battery cages that were randomly assigned to one of five treatment groups (17 replicates per treatment).

The treatments were:

(i) **CON**, a basal diet fed to uninfected chicks

(ii) **CON+SAL**, a basal diet fed to *S.* Enteritidis infected chicks

(iii) **ANTIBIO+SAL**, a basal diet supplemented with 200 mg oxytetracycline per kg feed (Virbac, Centurion, South Africa) fed to *S.* Enteritidis infected chicks

(iv) **CC+SAL**, a diet containing 10% *C. chloropyga* larvae meal fed to *S.* Enteritidis infected chicks

(v) **BSF+SAL**, a diet containing 10% *H. illucens* larvae meal fed to *S.* Enteritidis infected chicks

Diets were formulated to meet the minimum nutrient requirements supplied by the Ross 308 broiler guide (Aviagen, 2014). Birds received the starter diet for ten days, grower diet for 13 days and a finisher diet up until slaughter date. The control diets (CON+SAL, CON-NEG and ANTIBIO+SAL) contained full-fat soya bean meal and soya bean oilcake as a protein source (Table 6.1). To formulate the CC and BSF diet, 10% larvae meal was included in the diets by partly replacing the soya bean sources, pure amino acids as well as vegetable oil. Diets were mixed at Mariendahl experimental farm and were fed in a mash form on an *ad-lib* basis to the birds from day-zero to day-28.

Table 6.1 Ingredient and calculated nutrient composition of trial starter diets

Ingredients	Units	[1]CON*	[2]CC	[3]BSF
Maize	%	50.64	56.71	52.35
Soya bean (Full fat)	%	26.00	13.30	11.00
Soya bean (46%)	%	17.84	15.41	22.50
L-lysine (HCl)	%	0.266	1.191	0.306
DL methionine	%	0.382	0.251	0.336
L-threonine	%	0.120	0.102	0.098
Premix	%	0.250	0.250	0.250
Limestone	%	1.399	1.626	1.017
Salt	%	0.266	0.092	0.236
Mono-calcium phosphate	%	1.981	1.767	1.617
Sodium bicarbonate	%	0.133	0.304	0.288
Sunflower oil	%	0.727	0.000	0.000
Hermetia illucens larvae meal (full fat)	%	0.000	0.000	10.00
Chrysomya chloropyga larvae meal (full fat)	%	0.000	10.00	0.000
Calculated nutrient composition				
Dry matter	%	88.64	88.70	88.73
AMEn chick	MJ/kg	12.55	12.55	12.55
Crude protein	%	22.80	23.00	22.80
Crude fibre	%	3.436	3.487	3.815
Crude fat	%	7.721	7.146	8.193
Lysine	%	1.472	1.473	1.463
Methionine	%	0.711	0.652	0.690
Cysteine	%	0.384	0.423	0.465
Methionine + Cysteine	%	1.095	1.075	1.155
Threonine	%	0.986	0.988	0.984
Tryptophan	%	0.266	0.218	0.282
Arginine	%	1.536	1.555	1.549
Isoleucine	%	1.022	0.990	1.019
Leucine	%	1.952	1.929	1.945
Histidine	%	0.614	0.638	0.613
Phenylalanine	%	1.048	1.104	1.026
Tyrosine	%	0.845	0.834	0.863
Valine	%	1.127	1.159	1.184
Ash	%	4.394	4.355	4.324
Calcium	%	0.960	1.000	1.000
Total phosphorous	%	0.904	0.852	0.843
Available phosphorous	%	0.480	0.500	0.480
Sodium	%	0.160	0.230	0.198
Chloride	%	0.250	0.245	0.250
Potassium	%	0.950	0.795	0.878

[1]CON = control diet; [2]CC = diet containing 10% *C. chloropyga* larvae meal; [3]10%BSF = diet containing 10% *H. illucens* larvae meal;
*The same control diet was used for the CON-NEG, CON+SAL and ANTBIO diet, but 200mg oxytetracycline per kg feed was supplemented in the ANTIBIO diet
[6]AMEn = Nitrogen-corrected apparent metabolisable energy

Table 6.2 Ingredient and calculated nutrient composition of grower diets

Ingredients	Units	[1]CON*	[2]CC	[3]BSF
Maize	%	52.36	59.47	53.99
Soya bean (Full fat)	%	30.00	21.61	20.27
Soya bean (46%)	%	11.83	5.30	12.54
L-lysine (HCl)	%	0.119	0.086	0.160
DL methionine	%	0.297	0.202	0.197
L-threonine	%	0.165	0.060	0.045
Premix	%	0.250	0.250	0.250
Limestone	%	1.267	1.431	0.768
Salt	%	0.268	0.099	0.242
Mono-calcium phosphate	%	1.757	1.437	1.386
Sodium bicarbonate	%	0.133	0.044	0.146
Sunflower oil	%	1.546	0.000	0.000
Hermetia illucens larvae meal (full fat)	%	0.000	0.000	10.00
Chrysomya chloropyga larvae meal (full fat)	%	0.000	10.00	0.000
Calculated nutrient composition				
Dry matter	%	88.60	88.51	88.59
AMEn chick	MJ/kg	13.00	13.00	13.00
Crude protein	%	21.50	21.50	21.50
Crude fibre	%	3.394	3.499	3.863
Crude fat	%	9.248	8.638	9.821
Lysine	%	1.283	1.304	1.286
Methionine	%	0.613	0.588	0.541
Cysteine	%	0.370	0.407	0.453
Methionine + Cysteine	%	0.983	0.995	0.994
Threonine	%	0.986	0.894	0.892
Tryptophan	%	0.249	0.197	0.266
Arginine	%	1.446	1.446	1.468
Isoleucine	%	0.962	0.917	0.963
Leucine	%	1.873	1.840	1.876
Histidine	%	0.585	0.650	0.588
Phenylalanine	%	0.993	1.040	0.978
Tyrosine	%	0.788	0.758	0.800
Valine	%	1.067	1.085	1.127
Ash	%	4.080	4.007	3.969
Calcium	%	0.870	0.870	0.870
Total phosphorous	%	0.873	0.760	0.779
Available phosphorous	%	0.435	0.435	0.435
Sodium	%	0.160	0.160	0.160
Chloride	%	0.230	0.230	0.230
Potassium	%	0.900	0.735	0.833

[1]CON = control diet; [2]CC = diet containing 10% *C. chloropyga* larvae meal; [3]10%BSF = diet containing 10% *H illucens* larvae meal;
*The same control diet was used for the CON-NEG, CON+SAL and ANTBIO diet, but 200mg oxytetracycline per kg feed was supplemented in the ANTIBIO diet
[6]AMEn = Nitrogen-corrected apparent metabolisable energy

Table 6.3 Ingredient and calculated nutrient composition of finisher diets

Ingredients	Units	[1]CON*	[2]CC	[3]BSF
Maize	%	55.17	62.21	56.89
Soya bean (Full fat)	%	30.00	23.79	20.00
Soya bean (46%)	%	8.379	0.000	8.987
L-lysine (HCl)	%	0.096	0.040	0.147
DL methionine	%	0.254	0.162	0.210
L-threonine	%	0.033	0.029	0.016
Premix	%	0.250	0.250	0.250
Limestone	%	0.275	0.113	0.248
Salt	%	0.275	0.113	0.248
Mono-calcium phosphate	%	1.563	1.241	1.193
Sodium bicarbonate	%	0.127	0.028	0.143
Sunflower oil	%	2.676	0.818	1.163
Hermetia illucens larvae meal (full fat)	%	0.000	0.000	10.00
Chrysomya chloropyga larvae meal (full fat)	%	0.000	10.00	0.000
Calculated nutrient composition				
Dry matter	%	88.60	88.46	88.56
[4]AMEn chick	MJ/kg	13.39	13.39	13.39
Crude protein	%	20.00	20.00	20.00
Crude fibre	%	3.284	3.414	3.736
Crude fat	%	10.42	9.875	10.98
Lysine	%	1.173	1.173	1.175
Methionine	%	0.554	0.531	0.536
Cysteine	%	0.354	0.390	0.435
Methionine + Cysteine	%	0.908	0.921	0.971
Threonine	%	0.801	0.808	0.804
Tryptophan	%	0.229	0.176	0.244
Arginine	%	1.338	1.334	1.350
Isoleucine	%	0.891	0.844	0.886
Leucine	%	1.783	1.745	1.776
Histidine	%	0.550	0.614	0.550
Phenylalanine	%	0.928	0.973	0.906
Tyrosine	%	0.730	0.693	0.736
Valine	%	0.998	1.013	1.052
Ash	%	3.802	3.724	3.624
Calcium	%	0.790	0.790	0.790
Total Phosphorous	%	0.774	0.696	0.714
Available phosphorous	%	0.395	0.395	0.395
Sodium	%	0.160	0.160	0.160
Chloride	%	0.230	0.230	0.230
Potassium	%	0.841	0.673	0.768

[1]CON = control diet; [2]CC = diet containing 10% *C. chloropyga* larvae meal; [3]10%BSF = diet containing 10% *H. illucens* larvae meal;
*The same control diet was used for the CON-NEG, CON+SAL and ANTBIO diet, but 200mg oxytetracycline per kg feed was supplemented in the ANTIBIO diet
[6]AMEn = Nitrogen-corrected apparent metabolisable energy

6.2.4 Production parameters

Feed consumption was recorded on a cage basis at weekly intervals. Individual feed intake was calculated as an average of the cage after correcting for chicks slaughtered during sampling days and for mortality. Bodyweight of all birds in a cage was measured at placement and weekly after that. Mortality was recorded daily. Feed conversion ratio (FCR) was determined for the whole period, taking into account the total amount of feed intake per cage as well as the total amount of live weight gained per cage, including the weight gained by the birds slaughtered at each sampling time point.

6.2.5 Confirming the absence of Salmonella in birds before infection

On day five, ten birds were randomly selected from cages to confirm that the birds were free from *Salmonella*. Chicks were euthanised by cervical dislocation, both ceca were removed aseptically and placed in a sterile Sterilin™ homogenizing bag (Thermo Fisher Scientific, Massachusetts, USA) containing 10ml peptone buffered water [1% (m/v) peptone, 0.5% (m/v) NaCl, 0.35% (m/v) Na_2HPO_4, 0.15% (m/v) KH_2PO_4, pH 7.2). The ceca were dissected longitudinally and cut into pieces in the bag with a sterile pair of scissors and homogenised for five minutes using a stomacher (Interscience, St Nom la Bretêche, France). Homogenised samples were enriched by inoculating samples in tetrathionate brilliant green bile (TGB) enrichment broth and incubating at 37°C for 18 hours. Thereafter, enriched samples were plated on SS agar containing 0.004% (m/v) novobiocin and incubated at 37ºC for 24 hours. No *Salmonella* growth on SS agar plates after direct plating and enrichment confirmed that chicks were *Salmonella* free before being challenged.

6.2.6 Infection of broilers with Salmonella

Salmonella enterica subsp. *enterica* serovar Enteritidis A9 was grown in Luria-Bertani (LB) broth for eight hours at 37°C. Cells were collected by centrifugation (8000 x g for ten minutes), washed three times with sterile phosphate-buffered solution (PBS), and diluted with peptone buffer water to a concentration of 4.5×10^8 CFU/ml. On day nine and ten, the chicks in all the treatments except for the CON-NEG treatment group were manually orally challenged twice (day nine and day ten) with 200 µl of the *S.* Enteritidis A9 (4.5×10^8 CFU/ml) solution using a micropipette. Chicks in the CON-NEG group received sterile peptone buffered water instead of *Salmonella* to serve as a control. To prevent cross-contamination between infected birds and birds in the CON-NEG group, non-infected birds were placed in cages at the back of the broiler house. A foot bath with disinfectant was placed in the pathway leading to the cages of CON-NEG birds, and researchers wore clean gloves and overalls when working with the birds in the CON-NEG group.

6.2.7 Salmonella colonisation of the cecum

Salmonella colonisation in the cecum of the chicks was determined on day 11, 14, 21, 25 and 28 (day 1, 4, 11, 15 and 18 post-second infection). At each time point, the smallest chick of the same sex was selected and euthanised by cervical dislocation. Both ceca were removed aseptically and processed as described in section 5.2.4.

Due to the substantial amount of *Salmonella* that adheres to the epithelial wall of the cecum (Soerjadi *et al.*, 1981) and the large variation in cecal content between birds, both cecal tonsils were homogenised to loosen the *Salmonella* adhering to the epithelium. Log colony forming units (CFU) will thus be expressed as Log CFU/g cecal content (*Salmonella* colonies in one gram of ceca content) as well as Log CFU/ceca (*Salmonella* colonies in the entire ceca) to compensate for the dilution effect of the cecal content. Homogenised cecal content was serially diluted in peptone buffered water within three hours of sampling and plated on *Salmonella-Shigella* (SS) agar containing 0.004% (m/v) novobiocin and incubated at 37ºC for 24 hours. In addition to direct plating, the 10^{-1} dilution were used for the enrichment process as described in 5.2.4. The colony-forming units (CFU) of *Salmonella* in the ceca were obtained from the SS agar plates after direct plating. If no *Salmonella* was observed in a sample after direct plating, but the sample was positive for *Salmonella* after the enrichment process, it was assumed to contain 10^{1} CFU/ceca. If no *Salmonella* was present on the SS agar plates after both direct plating and enrichment, the sample was considered *Salmonella* free.

Salmonella isolates were identified by morphological (growth on SS agar) and biochemical characteristics (urease test), and identification was confirmed by DNA homology tests. To differentiate between *Salmonella* and *Proteus* on the SS agar plates, a urease test, as described by Christensen (1946), was used. Briefly, isolates were inoculated in urease broth [2% (m/v) urea, 0.95% (m/v) Na_2HPO_4, 0.91% (m/v) KH_2PO_4, 0.01% (m/v) yeast extract and 0.001% (m/v) phenol red, pH 6.8] and incubated at 37°C for 24 hours. *Salmonella* is urease negative, while *Proteus* is urease positive. Urease secreted by *Proteus* hydrolyses urea into ammonia and CO_2, alkalising the media. A pH shift is detected by the colour change of the yellow phenol red to a bright pink colour. Thus, *Salmonella* positive isolates did not alter the yellow colour of the media.

To confirm that isolates were indeed *S.* Enteritidis A9, DNA was isolated from randomly selected colonies from all the sampling days by using the Zymo DNA extraction kit (Zymo Research, Irvine, California) according to the manufacturer's instructions. The *16S rDNA* gene was amplified by PCR using DNA primers 8F: (5′-CACGGATCCAGACTTTGATYMTGGCTCAG-3′) and 1512R: (5′-GTGAAGCTTACGGYTAGCTTGTTACGACTT-3′), according to methods previously described (Neveling *et al.*, 2012). The *16S rDNA* gene was purified using the QIAquik PCR purification kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The *16S rDNA* gene was sequenced using the BigDye Terminator V3.1 sequencing kit (Applied Biosystems, Foster City, California), as per manufacturer instructions. Primers 8F, 1512R, 1100R (5′-AGGGTTGCGCTCGTTG-3′) and 520R (5′-ACCGCGGCTGCTGGC-3′) were used to sequence the *16S rDNA* fragment (Neveling *et al.*, 2012). MEGA7 software (Kumar *et al.*, 2016) was used to align and construct the *16S rDNA* gene. Basic Local Alignment Search Tool (BLAST) analysis (Altschul *et al.*, 1990) was performed to determine sequence similarity.

6.2.8 Serum interferon-gamma levels

Blood was collected on day 15 (five days post the second infection) from the brachial vein from one bird per cage to determine serum interferon-gamma (IFN-γ) levels. Blood was collected in a 1 ml BD Vacutainer® blood collection tube (Becton, Dickinson and Company, New Jersey, USA) containing a clot activator. Serum was collected by centrifugation (1300 x g for 15 minutes at 4°C). Serum IFN-γ levels were determined using the chicken IFN-γ ELISA Kit (Elabscience, Texas, USA), as per manufacturer's instructions.

6.2.9 Serum bactericidal activity

On day 11, 14, 21, 25 and 28, blood was collected during the slaughter process. Serum was separated by centrifugation at 1800 x g for ten minutes at 4°C and stored at -20°C until further processing. The serum bactericidal activity against *E. coli* and *Salmonella* was determined using a turbidimetric assay (Amadori *et al.*, 1997). Briefly, an overnight culture of *S.* Enteritidis A9 and *E. coli* DH5α was suspended separately in LB broth (1% v/v), incubated at 37ºC for six hours until the optical density at 590nm doubled, an indication that bacteria are in the exponential growth phase. The bacteria were serially diluted with sterile saline solution to a 1:100 dilution. Subsequently, 50 µl serum (in duplicate), together with 50 µl of Hanks's balanced salt solution (HBSS), 100 µl of LB broth and 10 µl of either *S.* Enteritidis A9 or *E. coli* DH5α bacterial suspension was added to a microtiter plate. Control wells with or without bacteria were set up in which serum and bacteria were replaced with HBSS at the same volumes. Plates were covered and incubated in a humidified incubator at 37ºC for 18 hours. Bacterial growth was quantified by reading the absorbance at 690 nm by means of a spectrophotometer (DU730 UV-Vis spectrophotometer, Beckman, California, USA) before and after incubation.

6.2.10 Serum lysozyme concentration

Serum lysozyme activity was assessed using a lyso-plate assay (Bonizzi *et al*., 1989), as described in Chapter 5. Briefly, serum samples were distributed into wells made into solidified agar containing a suspension of *Micrococcus lysodeikticus* (Sigma-Aldrich, St. Louis, MO, USA). Serum samples and egg white lysozyme standards (Sigma-Aldrich, St. Louis, MO, USA) were distributed in duplicate in the wells and placed in a humidified incubator at 37ºC for 18 hours. The diameter of the lysed areas around the wells was measured, and the lysozyme concentration was determined using a semi-logarithmic plot created from the diameters obtained from the wells containing the lysozyme standard concentration ranges.

6.2.11 Wing web thickness response to PHA-P (Lymphoproliferative response)

The proliferative response of T lymphocytes was assessed on day 24 from one bird per cage using the phytohaemagglutinin (PHA) mitogen test as previously described by Corrier & DeLoach (1990). Briefly, a dose of 0.1 mL of 1mg PHA-P (L8754, Sigma Aldrich, St. Louis, MO, USA) dissolved in 100 µl PBS was injected intradermally into the left-wing web (patagium) of the birds while the right-wing web received a control injection of 0.1 ml sterile PBS. The thickness of each wing web was measured in triplicate immediately before and 24 hours post-injection using a thickness meter with an accuracy of 0.01 mm. The wing web swelling reactions to PHA-P were calculated using Equation 6.1:

Equation 6.1

$$\text{Index} = (\text{mm post PHA injection} - \text{mm pre PHA injection}) - (\text{mm post PBS injection} - \text{mm pre PBS injection})$$

6.2.12 Haematological parameters

Blood was collected from one bird per cage on day 18 and 29 via the brachial vein into a 1 ml K_2-EDTA BD Vacutainer® blood collection tube (Becton, Dickinson and Company, New Jersey, USA). Automated

full blood counts, including erythrocyte counts and their related parameters, as well as total leukocyte count, were measured using the Celldyne 3700CS haematology analyser (Abbott Diagnostics, Illinois, USA).

6.2.13 Statistical analysis

Statistical analysis was performed using STATISTICA 64 version 11 (2012) for a completely randomised design. Data were tested for normality and homogeneity using Shapiro-Wilk's and Levene's test, respectively. One-Way analysis of variance (ANOVA) or Kruskal-Wallis tests was performed where appropriate. Fisher's LSD post hoc test was used when data were analysed through a one-way ANOVA, while Games Howell post hoc tests were performed when significance was determined by means of the Kruskal Wallis test. Differences were deemed to be significant at $P \leq 0.05$.

6.3 Results

6.3.1 Antibacterial activity of the peptide extracts

Extracts from the raw CC larvae as well as the oven-dried larvae meal had strong antimicrobial activity against *E. coli* at a concentration of 40 g/mg and 80 g/mg (Table 6.4). However, the inhibition against *Salmonella* was only moderate and concentration as high as 80 mg/ml was needed for inhibition. Extracts from BSF larvae and larvae meal exhibited an average inhibition against *E. coli* and a weak inhibition against *Salmonella*.

Table 6.4 Growth inhibition and antibacterial activity of the peptide extracts from *Chrysomya Chloropyga and Hermetia illucens* larvae and larvae meal

	C. chloropyga				*H. illucens*			
	Raw larvae		Larvae meal		Raw larvae		Larvae meal	
	80 mg/ml	40 mg/ml	80 mg/ml	40 mg/ml	80 mg/ml	40 mg/ml	80 mg/ml	40 mg/ml
E. coli	++++	++++	++++	+++	++	--	++	--
Salmonella	++	--	++	--	+	--	+	--

No inhibition: -- (Bacteria growth was similar to that of negative control)
Weak inhibition: +
Average inhibition: ++
Strong inhibition: +++
Very strong inhibition ++++ (bacteria growth was completely inhibited, similar to the positive control)

6.3.2 Production parameters

There were no differences for live weight between treatments at placement (Table 6.5). Live weight was significantly affected by dietary treatment by day seven and 14. Live weight of chicks in CC+SAL and BSF+SAL treatments were the heaviest (*P* <0.05) on day seven (before the *Salmonella* challenge) when compared to the antibiotic and control treatment groups. However, on day 14, only chicks in the CC+SAL treatment weighed significantly more than the CON+SAL and CON-NEG group, with the ANTIBIO+SAL and BSF+SAL groups being intermediate. No significant differences in live weight were observed on day 21 and 28. Chicks in the BSF+SAL group had significantly higher feed intake in the first week compared to the CON+SAL and ANTIBIO+SAL treatment groups, with the CC+SAL and CON-NEG groups being intermediate. No significant differences in feed intake were observed in weeks two, three and four (after the challenge), or for cumulative feed intake (day 0-28). The larvae meal diets significantly improved FCR (Table 6.5) of chicks in the CC+SAL and BSF+SAL and ANTIBIO+SAL treatment groups when compared to the CON+SAL groups (1.29, 1.29, 1.31, *vs* 1.35, respectively).

Table 6.5 The effect of dietary inclusion of *Hermetia illucens* or *Chrysomya chloropyga* larvae meal, or oxytetracycline, on the production parameters of *Salmonella* Enteritidis infected broiler chickens (mean ± s.e)

	Treatments[1]					
	CON	CON+SAL	CC+SAL	BSF+SAL	ANTIBIO+SAL	*P*-value
Live weight (gram)						
Day[2] 0	45 ± 0	45 ± 0	45 ± 0	45 ± 0	45 ± 0	0.590
Day[2] 7	148^{b} ± 2	145^{b} ± 1	159^{a} ± 1	163^{a}± 1	151^{b} ± 2	≤0.001
Day 14	372^{b} ± 12	368^{b} ± 10	419^{a} ± 8	408^{ab} ± 11	388^{ab} ± 8	0.002
Day 21	782 ± 33	791 ± 30	855 ± 20	841 ± 25	817 ± 23	0.116
Day 28	1456 ± 37	1441 ± 42	1558 ± 28	1537 ± 35	1517 ± 31	0.093
Intake (gram per period)						
Day 0-7	122^{ab} ± 2	119^{a} ± 1	124^{ab} ± 1	126^{b} ± 1	120^{a} ± 2	0.0017
Day 7-14	304 ± 13	304 ± 10	338 ± 11	306 ± 13	319 ± 8	0.1545
Day 14-21	527 ± 19	571 ± 21	600± 18	595 ± 11	578 ± 19	0.060
Day 21-28	974 ± 22	989 ± 34	958 ± 18	959 ± 26	984 ± 21	0.8602
Day 0-28	1935 ± 41	2013 ± 43	2018 ± 31	1988 ± 35	2001 ± 41	0.5277
FCR	1.32^{ab} ± 0.01	1.35^{a} ± 0.02	1.29^{b} ± 0.01	1.29^{b} ± 0.02	1.31^{b} ± 0.01	0.030

[1] CON-NEG = Non-infected broilers receiving control diet; CON+SAL = *Salmonella* infected broilers receiving control diet; CC and BSF+SAL = *Salmonella* infected broilers receiving diets containing 10% *C. chloropyga* larvae meal (CC+SAL) or 10% *H. illucens* larvae meal (BSF), ANTIBIO+SAL= *Salmonella* infected broilers receiving control diet supplemented with oxytetracycline
[2]Oral challenge with *Salmonella* only occurred on day nine and day ten.
[a,b] Means within rows with different superscripts differ significantly (*P* <0.05)

6.3.3 Effects of larvae meal on *Salmonella* colonisation in the ceca

As expected, no *Salmonella* was detected in the ceca of birds in the CON-NEG treatment group during any of the slaughter days, indicating that the facility was sterile and that no cross-contamination between cages/treatments occurred (Figure 6.1). One day after infection, birds in the ANTIBIO+SAL group had significantly lower mean CFU/g cecum content and CFU/ceca compared to the CON+SAL, CC+SAL and BSF+SAL group. On day 14 (three days post-challenge), chicks in the ANTIBIO+SAL treatment group still had significantly lower mean CFU/g counts than birds from the CON+SAL and BSF+SAL treatment groups, whereas birds from the CC+SAL treatment group showed a slight decrease in CFU counts, as birds from this group were intermediate to birds from the ANTIBIO+SAL and CON+SAL treatment groups. Compared to the CON-NEG group, mean CFU/g were still significantly higher in the CON+SAL and BSF+SAL treatment groups on all sampling days, whilst on day 21, CFU/g cecal content was significantly lower in the CC+SAL group compared to the CON+SAL group. An interesting phenomenon was that irrespective of the diet (except for CON), the CFU per gram weight in the ceca all decreased over time (Figure 6.1).

Salmonella counts were also expressed as Log CFU/ceca to account for the *Salmonella* adhering to the epithelium of the ceca. It was also included to compensate for the dilution effect of the cecal content since some ceca were full of digesta when the animals were slaughtered while some ceca were almost empty.

Considering the total amount of colony-forming units of *S.* Enteritidis in both cecal tonsils (Figure 6.2), the intake of CC meal had no effect on the concentrations of *Salmonella* on day 11 (one-day post-infection), as levels were similar to that of birds from the CON+SAL group and significantly higher than the CON-NEG chickens. *Salmonella* concentrations in the ceca of CC+SAL birds decreased from day 14 (four days post-infection), owing to concentrations intermediate to those in the CON-NEG and CON+SAL birds up until day 25. After 18 days of infection (day 28), birds receiving CC meal showed reduced (P <0.05) levels of *Salmonella* in their ceca as compared to birds from the CON+SAL group (0.8 *vs* 2.1 Log CFU, respectively). When considering the distribution of the number of birds classified according to *Salmonella* counts in both cecal tonsils (Figure 6.3), CON-NEG had no birds with any *Salmonella* whilst ANTIBIO+SAL had a significantly lower number of birds with low CFU counts per ceca on day 11 when compared with the CON+SAL group. On days 24 and 28, the CC+SAL treatment group was the only group intermediate to the CON+SAL and CON-NEG treatment groups.

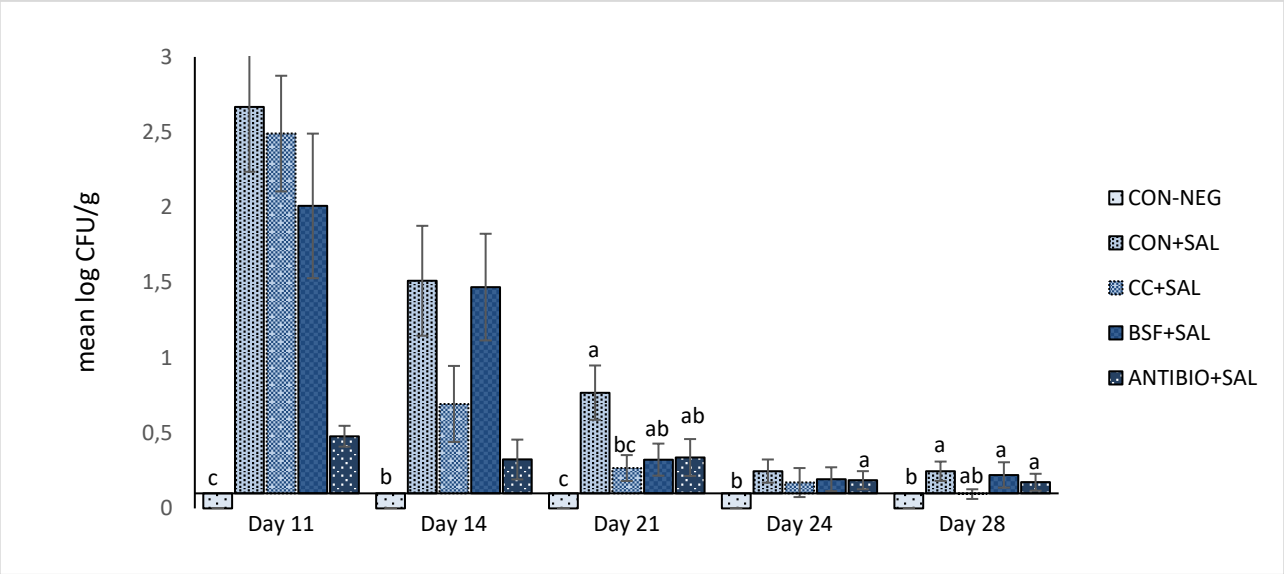

Figure 6.1 Mean log CFU of *Salmonella* per gram of ceca content of broilers infected with 200 µl (9 x 107 CFU) *S.* Enteritidis on day nine and day ten.
Treatment groups were: CON-NEG = Non-infected broilers receiving control diet; CON+SAL = *Salmonella* infected broilers receiving control diet; CC+SAL and BSF+SAL = *Salmonella* infected broilers receiving diets containing 10% *C. chloropyga* larvae meal (CC) or 10% *H. illucens* larvae meal (BSF), ANTIBIO+SAL= *Salmonella* infected broilers receiving control diet supplemented with oxytetracycline. [a,b,c] Bars with different superscripts within sampling day differ significantly (P <0.05). Error bars represents standard error of the mean.

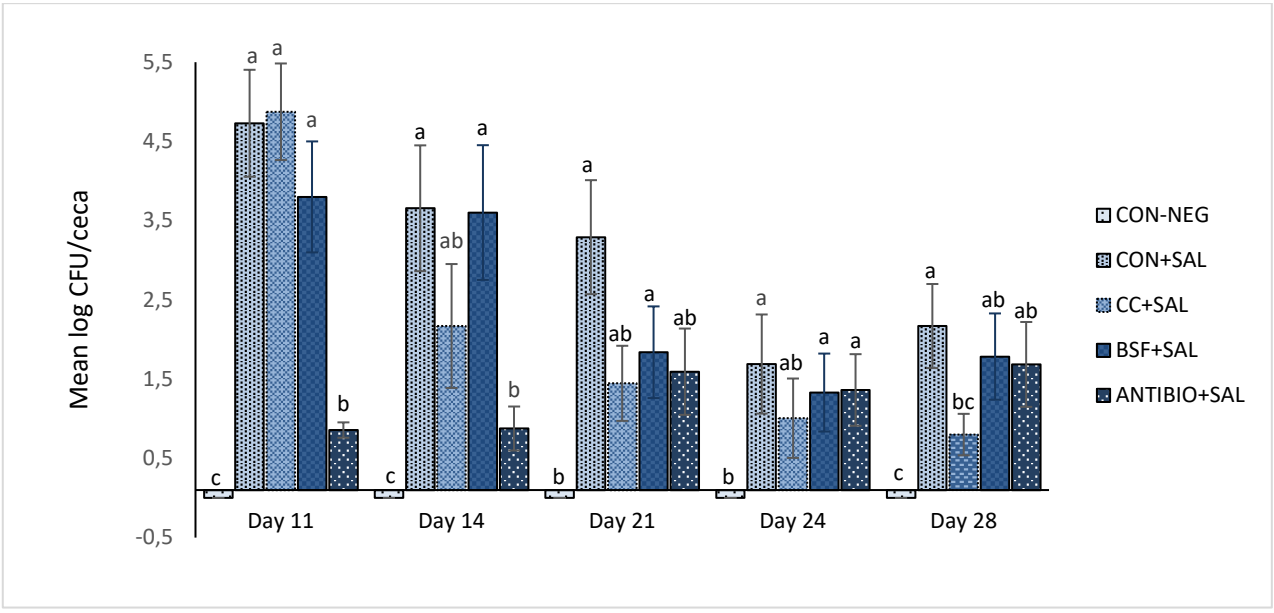

Figure 6.2 Total Mean log CFU of *Salmonella* in both ceca of broilers infected with 9 x 10^7 CFU *S.* Enteritidis on day nine and day ten.
Treatment groups were: CON-NEG = Non-infected broilers receiving control diet; CON+SAL = *Salmonella* infected broilers receiving control diet; CC+SAL and BSF+SAL = *Salmonella* infected broilers receiving diets containing 10% *C. chloropyga* larvae meal (CC) or 10% *H. illucens* larvae meal (BSF), ANTIBIO+SAL= *Salmonella* infected broilers receiving control diet supplemented with oxytetracycline. [a,b,c] Bars with different superscripts within sampling day differ significantly (P <0.05). Error bars represents standard error of the mean.

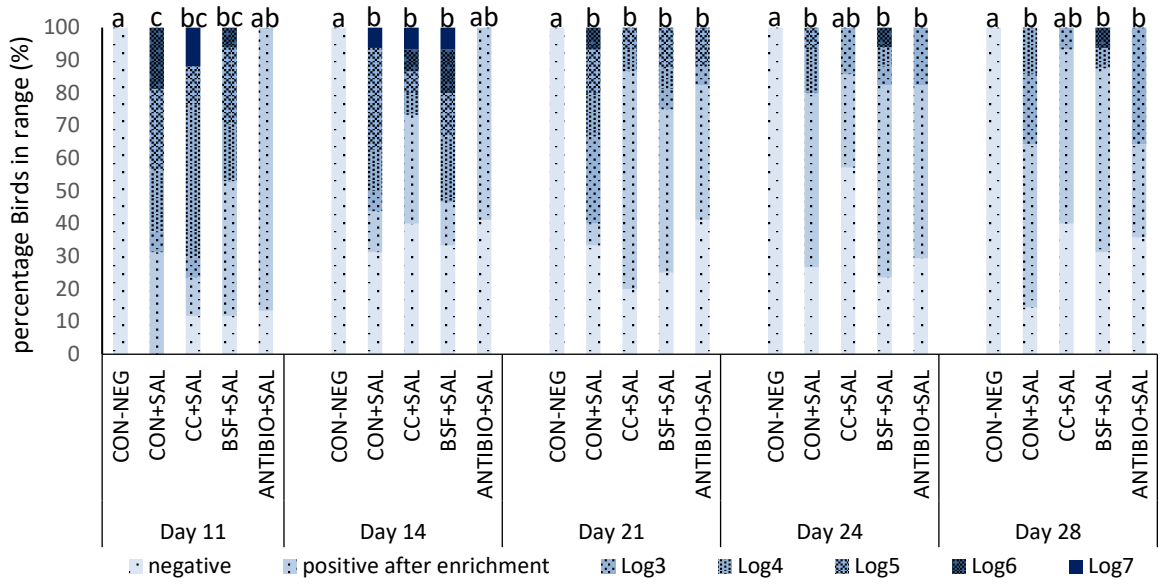

Figure 6.3 Representation of the percentage of birds per treatment classified according to bacterial counts in the ceca after infection with *Salmonella* Enteritidis on day nine and day ten.
Treatment groups were: CON-NEG = Non-infected broilers receiving control diet; CON+SAL = *Salmonella* infected broilers receiving control diet; CC+SAL and BSF+SAL = *Salmonella* infected broilers receiving diets containing 10% *C. chloropyga* larvae meal (CC) or 10% *H. illucens* larvae meal (BSF), ANTIBIO+SAL= *Salmonella* infected broilers receiving control diet supplemented with oxytetracycline. [a,b,c] Bars with different superscripts within sampling day differ significantly (P <0.05).

6.3.4 Wing web thickness index, serum lysozyme activity and serum IFN-γ concentrations

Phytohaemagglutinin-induced swelling (Figure 6.4) was significantly affected by treatment. It showed its lowest value for the CON+SAL and CON-NEG treatment groups (0.64, 0.57 respectively) and its highest values for the two larvae meal treatments (CC+SAL= 0.95; BSF+SAL= 1.08), with the ANTIBIO+SAL group (0.83) being intermediate to the CON+SAL and BSF+SAL group. Treatments significantly affected serum lysozyme concentrations on day 11 and 14. *Salmonella* infection and dietary antibiotic additives did not significantly alter lysozyme concentrations (Figure 6.5), as the serum lysozyme concentrations were similar for broilers in the CON-NEG, CON+SAL and ANTIBIO+SAL treatments groups on all the sampling days. However, on day 11, broilers receiving either larvae meal sources (CC+SAL = 11; BSF+SAL = 12.3 µg/ml) had significantly higher lysozyme concentrations than birds from the CON-NEG treatment group (11.8 µg/ml), with only CC+SAL broilers significantly differing from the CON+SAL treatment group (8.6 µg/ml). On day 14, CC+SAL broilers had significantly higher serum lysozyme concentrations compared to the CON, CON+SAL and ANTIBIO+SAL broilers, with BSF+SAL treatment group being intermediate to all the treatment groups. Serum IFN-gamma concentrations (Figure 6.6) in birds from the different treatment groups did not significantly differ on day 15 (five days post-infection).

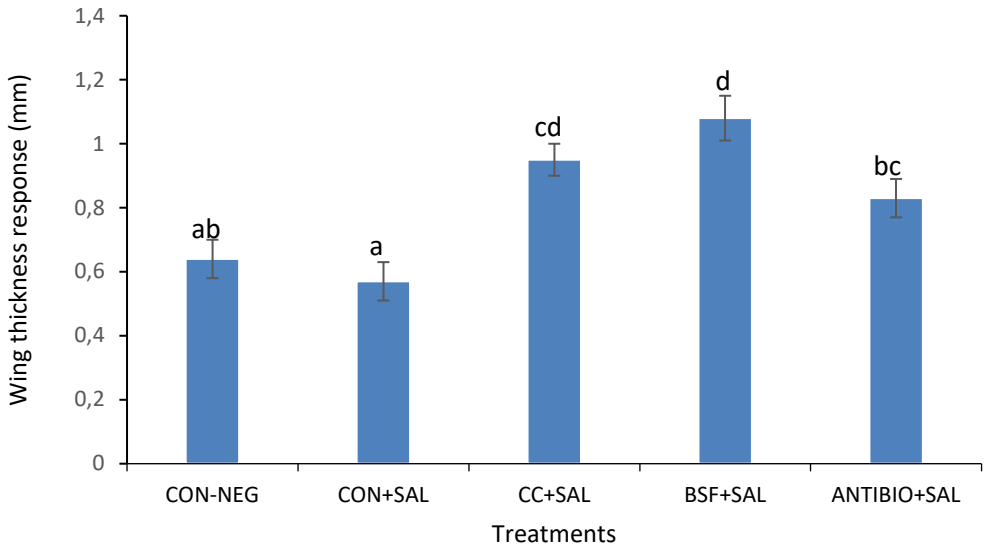

Figure 6.4 Mean wing thickness response to injection with phytohemagglutinin (PHA-P) on day 24.
Treatment groups were: CON-NEG = Non-infected broilers receiving control diet; CON+SAL = *Salmonella* infected broilers receiving control diet; CC+SAL and BSF+SAL = *Salmonella* infected broilers receiving diets containing 10% *C. chloropyga* larvae meal (CC) or 10% *H. illucens* larvae meal (BSF), ANTIBIO+SAL= *Salmonella* infected broilers receiving control diet supplemented with oxytetracycline. Error bars represents standard error of the mean.

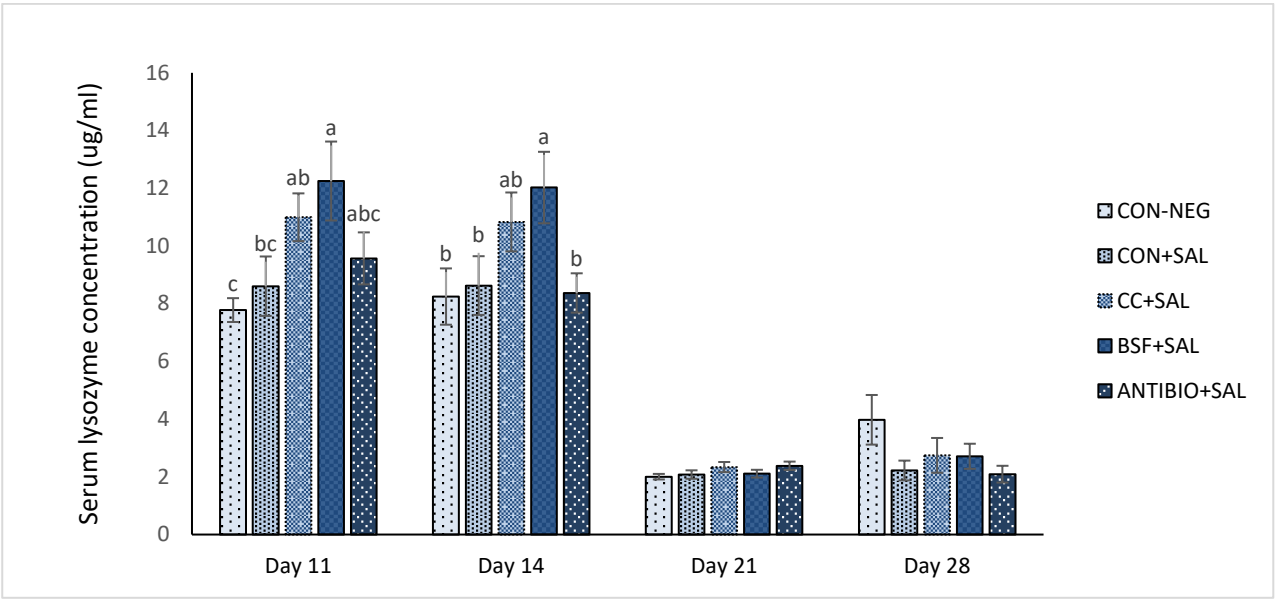

Figure 6.5 Mean serum lysozyme concentrations of broilers receiving different dietary treatments.
Treatment groups were: CON-NEG = Non-infected broilers receiving control diet; CON+SAL = *Salmonella* infected broilers receiving control diet; CC+SAL and BSF+SAL = *Salmonella* infected broilers receiving diets containing 10% *C. chloropyga* larvae meal (CC) or 10% *H. illucens* larvae meal (BSF), ANTIBIO+SAL= *Salmonella* infected broilers receiving control diet supplemented with oxytetracycline. ^{a,b,c} Bars with different superscripts differ significantly (P <0.05). Error bars represents standard error of the mean.

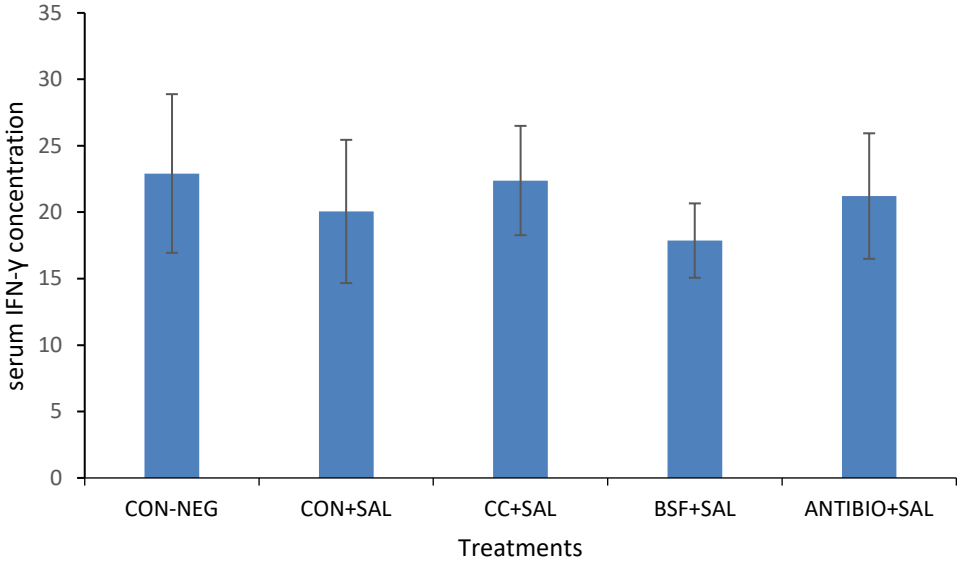

Figure 6.6 Mean serum IFN-γ concentrations measured on day 15 from broilers from different treatment groups. Treatment groups were: CON-NEG = Non-infected broilers receiving control diet; CON+SAL = *Salmonella* infected broilers receiving control diet; CC+SAL and BSF+SAL = *Salmonella* infected broilers receiving diets containing 10% *C. chloropyga* larvae meal (CC) or 10% *H. illucens* larvae meal (BSF), ANTIBIO+SAL= *Salmonella* infected broilers receiving control diet supplemented with oxytetracycline. Error bars represents standard error of the mean.

6.3.5 Serum bactericidal activity against *Salmonella* Enteritis and *Escherichia coli*

One day post-infection, birds from the CON-NEG treatment group expressed significantly lower serum bactericidal activity (Figure 6.7) against *Salmonella* when compared to birds from the CC+SAL, BSF+SAL and ANTIBIO+SAL treatment groups (28.6; 28.6; 28.0 *vs* 13.8%, respectively). Only a slight increase in activity was detected for the CON-NEG group (21.2%), resulting in values being intermediate to the CON-NEG and the other three groups. On day 21, only birds from the CC+SAL group expressed significant higher bactericidal activity compared to the CON-NEG group (16.2 *vs* 3.2%). No significant differences were observed between the treatment groups on day 14 and 28. Bactericidal activity against *E. coli* (Figure 6.8) significantly differed on day 14 between treatment groups. Interestingly, the opposite was observed with the activity against *Salmonella*, the challenged organism, with the control treatment groups expressing significantly higher bactericidal activity against *E. coli* than BSF+SAL and ANTIBIO+SAL treatment groups.

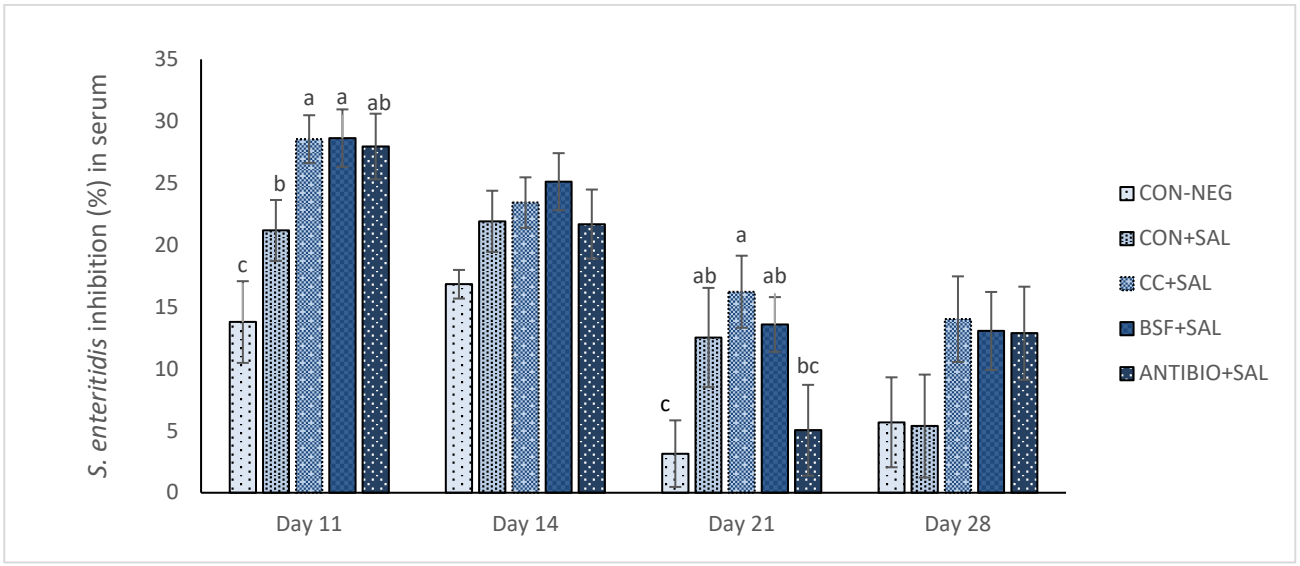

Figure 6.7 Inhibition percentage against *Salmonella* Enteritidis in the serum of broilers infected with 200 µl (9 x 10^7 CFU) *Salmonella* Enteritidis on day nine and day ten.
Treatment groups were: CON-NEG = Non-infected broilers receiving control diet; CON+SAL = *Salmonella* infected broilers receiving control diet; CC+SAL and BSF+SAL = *Salmonella* infected broilers receiving diets containing 10% *C. chloropyga* larvae meal (CC) or 10% *H. illucens* larvae meal (BSF), ANTIBIO+SAL= *Salmonella* infected broilers receiving control diet supplemented with oxytetracycline. [a,b,c] Bars with different superscripts within sampling day differ significantly (*P* <0.05). Error bars represents standard error of the mean.

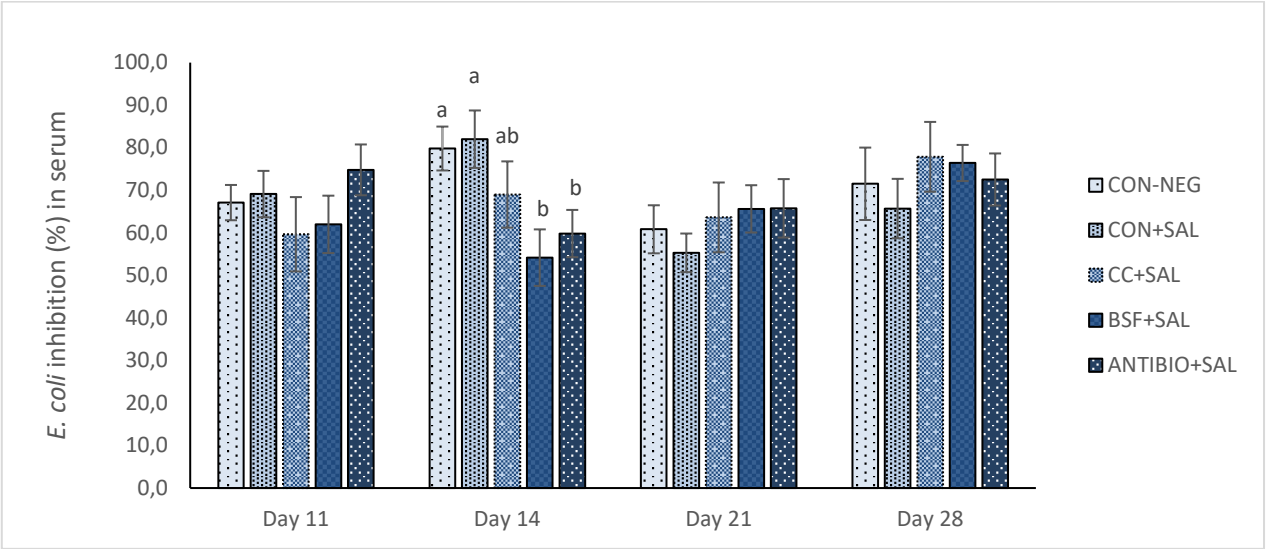

Figure 6.8 Inhibition percentage against *Escherichia coli* in the serum of broilers infected with 200 µl (9 x 10^7 CFU) *Salmonella* Enteritidis on day nine and day ten. Treatment groups were: CON-NEG = Non-infected broilers receiving control diet; CON+SAL = *Salmonella* infected broilers receiving control diet; CC+SAL and BSF+SAL = *Salmonella* infected broilers receiving diets containing 10% *C. chloropyga* larvae meal (CC) or 10% *H. illucens* larvae meal (BSF), ANTIBIO+SAL= *Salmonella* infected broilers receiving control diet supplemented with oxytetracycline. [a,b,c] Bars with different superscripts within sampling day differ significantly (*P* <0.05). Error bars represents standard error of the mean.

6.3.6 Haematological parameters and organ weights

Birds from the different treatment groups had similar relative lymphoid organs weights (Table 6.6) at all sampling days. Blood was collected eight days and 18 days post *Salmonella* infection. Haematological parameters of birds from the different treatment groups did not significantly differ on both sampling days (Table 6.7).

Table 6.6 The effect of dietary inclusion of dietary larvae meal or oxytetracycline on the lymphoid organ weights relative to the bodyweight of *Salmonella* Enteritidis infected broiler chickens (mean ± s.e)

	Treatments[1]					
	CON-NEG	CON+SAL	CC+SAL	BSF+SAL	ANTIBIO+SAL	P-value
Day 14						
Bursa %	0.218 ± 0.016	0.214 ± 0.017	0.237 ± 0.014	0.218 ± 0.011	0.243 ± 0.011	0.528
Spleen %	0.075 ± 0.004	0.082 ± 0.005	0.079 ± 0.005	0.080 ± 0.005	0.083 ± 0.004	0.712
spleen:bursa	0.366 ± 0.025	0.415 ± 0.036	0.349 ± 0.027	0.375 ± 0.024	0.351 ± 0.021	0.427
Day 21						
Bursa %	0.244 ± 0.012	0.234 ± 0.017	0.236 ± 0.018	0.218 ± 0.014	0.212 ± 0.015	0.541
Spleen %	0.084 ± 0.006	0.081 ± 0.005	0.083 ± 0.004	0.083 ± 0.004	0.079 ± 0.004	0.952
spleen:bursa	0.355 ± 0.032	0.363 ± 0.026	0.373 ± 0.024	0.402 ± 0.030	0.402 ± 0.036	0.706
Day 28						
Bursa %	0.228 ± 0.010	0.229 ± 0.013	0.252 ± 0.015	0.254 ± 0.014	0.220 ± 0.019	0.372
Spleen %	0.087 ± 0.005	0.092 ± 0.005	0.101 ± 0.007	0.096 ± 0.004	0.083 ± 0.004	0.114
spleen:bursa	0.384 ± 0.021	0.419 ± 0.037	0.421 ± 0.041	0.403 ± 0.036	0.414 ± 0.034	0.936

[1] CON-NEG = Non-infected broilers receiving control diet; CON+SAL = *Salmonella* infected broilers receiving control diet; CC+SAL and BSF+SAL = *Salmonella* infected broilers receiving diets containing 10% *C. chloropyga* larvae meal (CC) or 10% *H. illucens* larvae meal (BSF), ANTIBIO+SAL= *Salmonella* infected broilers receiving control diet supplemented with oxytetracycline

Table 6.7 The effect of dietary inclusion of dietary larvae meal or oxytetracycline on the haematological parameters of *Salmonella* Enteritidis infected broiler chickens (mean ± s.e)

	Treatments[1]					
	CON-NEG	CON+SAL	CC+SAL	BSF+SAL	ANTIBIO+SAL	P-value
Day 18						
WBC	19.7 ± 0.36	18.3 ± 0.77	18.1 ± 0.82	18.2 ± 0.86	18.3 ± 0.97	0.696
RBC	2.26 ± 0.04	2.32 ± 0.04	2.24 ± 0.04	2.30 ± 0.04	2.29 ± 0.03	0.625
HGB	13.4 ± 0.23	13.6 ± 0.21	13.3 ± 0.18	13.6 ± 0.23	13.4 ± 0.20	0.873
MCV	91.7 ± 0.66	90.9 ± 0.71	91.6 ± 0.86	91.6 ± 0.41	91.0 ± 0.93	0.924
RDW	12.5 ± 0.16	12.4 ± 0.20	12.4 ± 0.25	12.6 ± 0.16	12.6 ± 0.27	0.948
PCV	21.1 ± 0.33	21.1 ± 0.36	20.5 ± 0.36	21.1 ± 0.37	20.8 ± 0.35	0.629
MCH	59.3 ± 0.31	58.8 ± 0.41	59.5 ± 0.54	59.1 ± 0.40	58.8 ± 0.45	0.737
MCHC	63.8 ± 1.55	64.7 ± 0.49	65.2 ± 0.62	64.5 ± 0.36	64.6 ± 0.37	0.819
Day 28						
WBC	21.3 ± 0.50	21.2 ± 0.37	21.2 ± 0.41	21.5 ± 0.43	20.8 ± 0.33	0.806
RBC	2.34 ± 0.04	2.41 ± 0.04	2.35 ± 0.05	2.37 ± 0.05	2.40 ± 0.05	0.449
HGB	13.8 ± 0.18	14.3 ± 0.20	14.1 ± 0.22	13.8 ± 0.22	14.1 ± 0.27	0.534
MCV	88.2 ± 0.60	87.5 ± 0.66	88.6 ± 0.80	87.7 ± 0.63	86.6 ± 0.77	0.454
RDW	12.2 ± 0.19	12.1 ± 0.25	12.5 ± 0.24	12.2 ± 0.13	12.3 ± 0.14	0.611
PCV	20.6 ± 0.40	21.0 ± 0.28	20.8 ± 0.43	20.7 ± 0.40	20.8 ± 0.42	0.962
MCH	59.2 ± 0.50	59.3 ± 0.57	59.9 ± 0.62	58.5 ± 0.38	58.6 ± 0.58	0.333
MCHC	67.1 ± 0.62	67.7 ± 0.57	67.7 ± 0.69	66.8 ± 0.45	67.7 ± 0.44	0.658

[1] CON-NEG = Non-infected broilers receiving control diet; CON+SAL = *Salmonella* infected broilers receiving control diet; CC+SAL and BSF+SAL = *Salmonella* infected broilers receiving diets containing 10% *C. chloropyga* larvae meal (CC) or 10% *H. illucens* larvae meal (BSF), ANTIBIO+SAL= *Salmonella* infected broilers receiving control diet supplemented with oxytetracycline
WBC = Leukocyte count; RBC = Erythrocyte count; HGB = Haemoglobin; MCV = Mean corpuscular volume; RDW = Erythrocyte distribution width; PCV = packed cell volume / haematocrit; MCH = mean cell haemoglobin, MCHC = Mean cell haemoglobin concentrations

6.4 Discussion

6.4.1 Production parameters

The inclusion of *H. illucens* (BSF) and *C. chloropyga* (CC) larvae meal in broiler diets showed satisfactory production results during this trial (Table 6.5). Partly substituting soya bean meal with either BSF or CC larvae meal in broiler diets resulted in increased live weight during the starter phase. The effects on live weight diminished over time as no differences were observed between birds from the different treatment groups at slaughter age. Several researchers reported an increase in live weight during the starter and grower phase when ten to 15% BSF larvae meal were added to maize-soya-based broiler diets (Téguia *et al.*, 2002;

Dabbou *et al.*, 2018; Velten *et al.*, 2018), whereas Onsongo *et al.* (2018) noted that the partial replacement of fishmeal and soya bean meal with BSF larvae meal did not affect the live weight of broilers.

Dietary inclusion of either larvae meal sources (CC or BSF) to a plant-based diet significantly improved total FCR in the current trial. Increased FCR's are not always reported in broilers fed BSF larvae meal, the lack of improved FCR are more often observed when larvae meal are used to replace fishmeal in broiler diets (Téguia *et al*.,2002; 2018; Onsongo *et al*., 2018). In general, broiler performance improves when receiving a small percentage of dietary fishmeal in plant-based diets (Mikulec *et al.*, 2004; Ojewola *et al.*, 2005). Interestingly, Oluokun (2000) and Pretorius (2011) also demonstrated that fishmeal has certain production benefits when compared to maize-soya based diets. Nonetheless, the inclusion of BSF or *M. domestica* larvae meal in maize-soya-based diets mimics the effects fishmeal has on FCR and average daily gain (Oluokun, 2000; Pretorius, 2011). Therefore, a greater production response can be expected when larvae meal is included in maize-soya based diets compared to maize-soya-fishmeal based diets.

In the current trial, the diets were not only iso-nitrogenous and iso-caloric but were formulated to fit the ideal amino acid profile of broilers, keeping the amino acid profile of all the diets similar thereby avoiding antagonism of amino acids and additional metabolic cost of deamination between treatment groups. Therefore, it is assumed that the improvement in FCR for chicks fed larvae meal diets were not due to energy, protein, or amino acid composition of the diets, but rather due to other unidentified growth factors or due to improved health. To identify the precise compounds responsible for improved growth is difficult; however, chitin present in the exoskeleton of the larvae might be a beneficial compound. Even though it is generally believed that chitin is indigestible, researchers recently reported the presence of chitin degradable enzymes in the proventriculus of chickens (Tabata *et al*., 2017). Interestingly, Ramachandran Nair *et al*. (1987) reported an increase in broiler growth and FCR when prawn shell-derived chitin was included in broiler diets. Even though *Salmonella* had no effect on broiler growth or FCR in the current study, increased immune responses due to dietary larvae meal (as demonstrated in Chapter 4 and 5) could indirectly improveFCR if there is an underlying subclinical disease or infection causing immunosuppression which impairs production parameters.

Lastly, oxytetracycline was used as an antibiotic feed additive in the current trial to determine if larvae meal can replace antibiotics as a growth promoter and to control colonisation of pathogens such as *Salmonella*. During this study, oxytetracycline served its purpose as a growth promotor by improving the FCR of broilers. Similar results have been reported by others (Emborg *et al.*, 2001; Kalavathy R *et al.*, 2008; Zulkifli *et al.*, 2010).

6.4.2 Salmonella colonisation in the ceca

Dietary inclusion of BSF larvae meal had no effect on cecal *S.* Enteritidis levels over the whole trial period, but long-term intake of CC larvae meal showed the potential in decreasing the cecal levels. The factors contributing to the clearance of *Salmonella* from the ceca of CC+SAL treatment chickens can be argued. Granted that chitin, together with chitooligosaccharides (a product of chitin digestion in the proventriculus of chickens (Tabata *et al.*, 2017) displays antimicrobial properties *in vitro* (Tsai *et al.*, 2000; Benhabiles *et al.*, 2012), it can be speculated that chitin aided in reducing *Salmonella* concentrations in CC+SAL chickens. However, given that CC*,* as well as BSF larvae, contains chitin in their exoskeleton, the factor contributing to

the *Salmonella* reduction in the CC+SAL treatment birds might not be the chitin after all. Furthermore, lauric acid and butyric acid are fatty acids known for their anti-*Salmonella* properties (Hoffman *et al.*, 2001; Van Immerseel *et al.*, 2004). However, despite BSF larvae containing high levels of lauric acid (Chapter 5) and/or its ability to increase butyrate production in the ceca (Borrelli *et al.*, 2017), these fatty acids did not reduce cecal *Salmonella* levels in broilers fed BSF larvae meal.

A heightened immune response in birds fed CC larvae meal could be responsible for the enhanced clearance of *Salmonella*; however, immune parameters measured during this study were similar for birds receiving BSF or CC meal. Nevertheless, it is possible that unidentified factors such as peptides and oligosaccharides in CC larvae meal might stimulate a more effective immune response via the gut-associated lymphoid tissue (GALT). Lastly, peptide extracts from CC and BSF larvae meals indicated that peptide extracts from CC larvae had higher antimicrobial activity against *Salmonella* compared to extracts from BSF larvae (Table 6.4). Even though the BSF genome encodes for 52 antimicrobial peptides (Moretta *et al.*, 2020), research shows that BSF larvae immunized with bacteria exhibits a greater expression of AMPs (Choi *et al.*, 2018; Lee *et al.*, 2020). It is possible that the CC larvae used in this study were more exposed to *Salmonella* in the animal offal substrate than BSF larvae reared on chicken waste, contributing to a higher expression of AMPs against *Salmonella* in CC larvae. Therefore, the slow decrease in *Salmonella* in the ceca over time in the CC+SAL groups can be a result of higher AMP levels in the CC larvae meal treatment. This, together with increased immune responses, could have resulted in significantly lower *Salmonella* concentrations in the ceca of CC+SAL broilers at day 28 when compared the CON+SAL treatment group. These aspects warrant further research to clarify the postulations above.

Regardless of reports illustrating changes in cecal microbiota of chickens after the intake of larvae meal (Borrelli *et al.*, 2017; Moula *et al.*, 2018), published data on the effect of BSF meal on pathogen colonisation in chickens is limited; and no published work could be found which demonstrates the antimicrobial properties of CC larvae meal. Direct comparisons with literature are therefore limited. However, Lee *et al.* (2018) reported a decrease in *Salmonella gallinarum* numbers in the liver, spleen, bursa and cecum of infected broilers receiving 3% dietary BSF larvae meal. Findings by Islam & Yang (2017) demonstrated that mealworm and super mealworm larvae meal (fermented with probiotics) possessed the ability to reduce *Salmonella* and *E. coli* colonisation in broilers. Likewise, Zhou *et al.* (2014) reported a decrease in *S. pullorum* in the faeces of chickens when they were administered with antimicrobial peptide extracts from house fly larvae

6.4.3 Immune parameters and lymphoid organ weights

To get a broad idea if larvae meal has immunomodulatory effects when fed to broilers infected with *Salmonella*, measurements of the general status of the immune system were made to determine immunosuppression or immunocompetence in broilers in response to a bacterial challenge. The bursa of Fabricius and the spleen are lymphoid organs that form part of the avian immune system (Yegani & Korver, 2008). The effective development of these organs is very important for optimal immune responses (Kwak *et al.,* 1999) and differences in relative lymphoid organ weights in an environmentally controlled trial can be an indication of immunocompetence. In the current study, lymphoid organ weights were not affected by *Salmonella* infection or dietary treatments. Similarly, Onsongo *et al.*, (2018) reported no differences in relative

spleen weight when 10% BSF meal was added to broiler diets. Similar to the larvae meal treatments, the use of oxytetracycline had no effect on the organ weights. This supports the findings of Al-Ankari & Homeida (1996), who found no differences in relative lymphoid organs weights after 25 days of oxytetracycline administration. On the other hand, a shrinkage in relative bursa weight was reported when the antibiotic was administered for 50 days, indicating that the prolonged feeding of oxytetracycline could have immune-suppressing effects (Al-Ankari & Homeida, 1996).

Interestingly, Al-Ankari & Homeida (1996) also noted a decrease in serum lysozyme concentrations after 50 days of oxytetracycline administration to broilers, yet again indicating that the prolonged feeding of oxytetracycline could have immune-suppressing effects. However, in the currents study, oxytetracycline supplementation did not alter serum lysozyme concentrations on any of the sampling dates, nor did infection with *Salmonella* have any effect. Lysozymes are key factors of the animal innate immune system that kills bacteria that invaded the host. This enzyme is a component of the secretory and phagocytic granules of neutrophils and is also produced by monocytes, macrophages, and epithelial cells. In the current trial, serum lysozyme activity was enhanced in birds fed BSF and CC larvae meals for 11 days. After 14 days of infection, lysozyme activity in birds from the CC+SAL treatment group marginally decreased, and only chicks fed BSF larvae meal had significantly higher activity compared to the antibiotic and control treatment groups. Similar results were observed when BSF larvae meal were fed to broiler quails (Chapter 5). As far as could be ascertained, the effect of larvae meal on serum lysozyme activity in chickens has not yet been determined; however, various authors had reported increased serum lysozyme concentrations when insects were fed to fish (Ming *et al.*, 2013; Henry *et al.*, 2018).

The exact mechanism/element in insect meal responsible for the enhanced lysozyme activity is unclear; however, chitin could once again be responsible for this phenomenon. Not only does chitin and partly deacetylated chitin serve as substrates for lysozymes (Amano & Ito, 1978; Pangburn *et al.*, 1982), but chitin supplementation has been shown to increase serum lysozyme activity (Harikrishnan, 2012). In addition, chitin increases monocyte and neutrophil levels, subsequently expanding the potential for lysozyme production (Harikrishan, 2012). Another possible explanation for increased lysozyme activity could be due to various bioactive substances produced by insects. For example, Dipterose, a polysaccharide extracted from insects, induces the activation of the protein complex NF-κBp65 in macrophages (Ohta *et al.*, 2014), which in turn activates the expression of lysozyme (Phi Van, 1996).

In addition to lysozymes, the blood of the host contains cellular components with phagocytic abilities that kill bacteria they come in contact with, contributing to the innate immune response. When considering serum bactericidal activity against *Salmonella*, a quick innate immune response was provoked in all the birds infected with *Salmonella*. However, oxytetracycline supplementation and both sources of dietary larvae meal gave rise to an amplified bactericidal competence against *Salmonella* in the host on day 11 (one-day post-infection). A quick response in the plasma was expected as *Salmonella* colonisation in organs already peaks three days after infection (Van Immerseel *et al.*, 2002). Serum bactericidal activity against *Salmonella* decreased with time, as did the concentrations of *Salmonella* in the ceca. It can be speculated that the response is only induced as needed. On day 21, the serum of the BSF+SAL, CC+SAL and CON+SAL chickens still exhibited higher *Salmonella* bactericidal activity compared to the CON-NEG chickens. Keeping in mind that the cecal *Salmonella* concentrations of the CON+SAL treatment were significantly higher than the

CC+SAL chickens on that day, a high *Salmonella* bactericidal response could be expected in the serum of CON+SAL chickens, owing to values similar to the CC+SAL group.

In response to flagellar antigens on *Salmonella*, CD4+ T lymphocytes produce IFN-γ (McSorley *et al.*, 2000), which in turn activates macrophages to induce NO production (Okamura *et al.*, 2004). It is believed that NO suppresses lymphocyte proliferation (Eisenstein & Hilburger, 1998) which may lead to a reduced toe web swelling after PHA-P mitogen injection in *Salmonella* infected birds. Correspondingly, Arnold and Holt (1995) noticed almost a complete lack of lymphocyte proliferation in response to a T lymphocyte mitogen in *Salmonella* infected chicks. However, in the current trial, the cellular immune response measured by PHA-induced swelling was not affected by *Salmonella* infection, but the dietary inclusion of larvae meal did however, increase this response. The specific action of the larvae meal on the cellular immune response is unclear and can be due to various reasons. For example, Cuesta *et al.* (2003) demonstrated that the main cellular innate immune activities could be enhanced in the presence of chitin.

Chitin and chitosan have the ability to activate Natural Killer cells to express pro-inflammatory cytokines such as interferon-gamma (IFN-γ), which further primes macrophages and enhances its oxidative burst (Shibata *et al.*, 1997). Even though the involvement of IFN-γ in the clearance of *Salmonella* infection is well documented (Lalmanach & Lantier, 1999), infection with *S.* Enteritidis in the current study had no effect on serum IFN-γ concentrations. Despite reports indicating increased plasma IFN-γ levels after peritoneal infection with *Salmonella* (Kumazawa *et al.*, 1991), elevated IFN-γ mRNA are found in the gut-associated lymphoid tissue in animals orally challenged with *S. typhimurium* (Ramarathinam *et al.*, 1991), suggesting that infection induces the releases of pro-inflammatory cytokines at the site of infection. Similar to the *Salmonella* treatment in this study, treatments containing larvae meal or oxytetracycline had no effect on serum IFN-γ levels. Although no published literature could be found on the impact of BSF or CC larvae meals on IFN-γ production in broilers, Kar (2017) noticed an increase in serum IFN-γ levels when yellow mealworms were fed to mice, but did not observe the same effect when BSF larvae meal was fed to pigs.

Haematological parameters

The study of haematological changes can be used as a diagnostic tool to assess health status since blood is an indicator of the physiological condition of animals (Da Cuña *et al.*, 2011). Haematological parameters can be altered due to environmental (Mohamed *et al.*, 2012), nutritional (Hackbarth, 1983) and pathological stress (Bossink *et al.*, 1999). The dietary inclusion of larvae meal or experimental *Salmonella* infection did not affect haematological parameters in the current study. Correspondingly, previous studies reported no effects on these traits after dietary inclusion of BSF larvae meal (Marono *et al.*, 2017; Wallace *et al.*, 2017), or insect meal from other species (Bovera *et al.*, 2015; Anggraeni *et al.*, 2016). Likewise, no differences in haematological traits were observed when birds were infected with *Salmonella* or supplemented with oxytetracycline in the current study, supporting the findings of previous authors (Shlosberg *et al.*, 1996; Alonge *et al.*, 2017).

The red blood cell count, white blood cell count and haemoglobin values in the current study (Table 6.7) were similar to values for ROSS 308 broiler chicks at their specific age group (Talebi *et al.*, 2005). Then again, values for haematocrit (PCV) were ±21% lower than the normal values for Ross broilers, while mean

corpuscular volume were ±39 FL lower than the standard usually reported (Scheele, Van Der Klis, Kwakernaak, Buys, & Decuypere, 2003; Toghyani, Tohidi, Gheisari, & Tabeidian, 2010). Haematocrit values <30% for the two age groups can be classified as anaemic (Shlosberg *et al.*, 1996; Talebi *et al.*, 2005). The reason for the low haematocrit values in the current study is unclear, as several environmental factors such as temperature, water intake, dietary copper and dietary iron can affect these parameters (Kubena *et al.*, 1972). Provided that the values are similarly low between the treatment groups, it can be concluded that the cause is not treatment related. Moreover, haematocrit is also an index of toxicity in the blood, with high levels suggesting the presence of toxic factors (Pikula *et al.*, 2010; Akinola & Etuk, 2015). Therefore, due to similar haematocrit values between treatments, dietary larvae meal showed no signs of toxicity when considering haematological indexes, nor did it compromise the health status of the animal.

6.5 Conclusion

The present study provides insight into the potential benefits dietary BSF and CC larvae meal could have during *Salmonella* infection in broilers. The use of either larvae meal sources resulted in improved FCR's, similar to birds receiving antibiotic growth promoters (oxytetracycline). Although BSF larvae meal did not decrease *Salmonella* colonisation, both larvae meal sources were effective in enhancing certain immune parameters, without negatively affecting growth performance. The use of oxytetracycline was most effective in lowering *Salmonella* levels during the first days after infection, but CC meal decreased *Salmonella* levels in the cecum in a slow but effective manner, resulting in significantly lower *Salmonella* counts at slaughter age. Results suggest that CC meal might reduce the risk of carcass contamination with *Salmonella* by lowering the levels present in the gastrointestinal tract and can be used as an alternative to antibiotic growth promotors to improve immunocompetence and growth efficacy.

6.6 References

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General Conclusion

Major foci have been placed on insects as a new sustainable protein source. One of the most widely researched insects for this purpose is larvae from *Hermetia illucens* flies (black soldier fly or BSF). This species is popular due to its ability to efficiently convert organic matter into a valuable protein and fat source. The research on the use of *C. chloropyga* (CC) larvae, a carrion blow fly which is excellent in converting animal offal, is scarce. Even though there are published data on the immunomodulatory and antimicrobial properties of BSF larvae (meal, no published data exist on these properties of CC larvae meal. This study aimed to evaluate the potential benefits of BSF larvae and CC larvae meal when used in poultry diets. The objectives of this study were to determine if the meal from CC larvae and BSF larvae (reared on different substrates) can be successfully used in broiler and quail diets and to determine the potential immunomodulatory and antimicrobial properties of these insect meals.

Results from this study demonstrated that that CC larvae meal reared on animal offal contained a high crude protein level of 60.8% (DM basis) and medium fat content of 23.7%. The protein content of CC larvae meal was almost double the amount of the protein compared to BSF larvae that were reared on either kitchen waste, chicken feed or chicken feed and animal fish offal (Chapter 3). It should be noted that for this study, protein in the larvae meal was not corrected for nitrogen in the chitin, therefore the true protein content of the larvae meals may vary slightly. When considering the amino acid requirements of broilers, the amino acid profile of BSF larvae meal reared on kitchen waste will provide the essential amino acid levels closest to the requirements, whereas an oversupply of essential amino acids will be most prominent in BSF larvae reared on kitchen waste (Chapter 3). Methionine and lysine are usually the first and second limiting amino acids in maize-soya-based poultry diets. Since CC larvae meal is high in methionine and lysine, its use in broiler diets will reduce the need to supplement the diets with these amino acids. A small acceptability trial was performed to determine when broilers have a choice, will they consume CC and BSF larvae meals? The outcome of the trial was positive (Chapter 3), and it was concluded that based on the nutrient profile of the larvae meal sources and its acceptability by broilers, CC and BSF larvae meals have the potential to be used as alternative protein sources in broiler diets.

Results indicated that BSF and CC larvae meals could be added to broiler and quail diets at a 10% inclusion level without a negative effect on production parameters (Chapter 3, 4 & 5). When broilers received 15% BSF larvae meal, a negative impact on FCR was recorded on day 35 (Chapter 4). Higher inclusion levels are not recommended since several studies indicate a reduction in protein digestibility and adverse effect on production parameters when high levels of BSF meal or chitin are used in monogastric animal diets (Hansen *et al.*, 2010; Kroeckel *et al.*, 2012; Karlsen *et al.*, 2017; Bovera *et al.*, 2018; Dabbou *et al.*, 2018). Even though no difference in FCR was observed in this study when healthy broilers or quails received larvae meal, there is an indication that larvae meal could improve FCR in infected animals, since BSF larvae and CC larvae meals improved FCR in *Salmonella* infected broilers (Chapter 6). Interestingly, the two larvae meal sources similarly improved FCR compared to the antimicrobial growth promoter (oxytetracycline) that were used in this study (Chapter 6). It is therefore possible that 10% larvae meal can replace the use of antibiotic growth promoters in broiler diets since it delivered similar results in challenged animals. This finding requires more research to gain insight into the mechanisms that produced these results

Even though studies on the effect of insect meal on antibody production is limited, there are certain factors such as polyphenols and chitin that are present in insects (Janssen *et al*., 2019) that stimulates antibody production (Koide, 1998; Taira *et al*., 2015). Results from this study indicate that both CC and BSF larvae meals have a positive effect on the humoral immune responses in poultry. Higher antibody titers were found in broilers receiving dietary CC and BSF larvae meals (Chapter 4), and in quails that were fed meal from BSF larvae reared on chicken feed (Chapter 5). The injection of phytohemagglutinin-P (PHA-P) induced proliferation of T-lymphocytes which resulted in a swelling response due to increased basophils and macrophage infiltration in the site of injection. There is an indication that larvae meal provoked a greater cellular immune response since the broiler chickens and quails receiving 10% CC or BSF larvae meals had a greater response to phytohemagglutinin-P injections (Chapter 4, 5, & 6).

Literature suggests that the antimicrobial peptides and lauric acid in dietary larvae meal will have antimicrobial effects against Gram-positive bacteria. Another essential compound effective against Gram-positive bacteria are lysozymes in the serum of the animals. Results from this study proved dietary larvae meal from both Dipteran species increases serum lysozyme concentrations in both broilers and quails. Both CC and BSF larvae meal were especially effective in increasing lysozyme concentrations in broilers during the starter phase (Chapter 4), whereas BSF larvae reared on fish offal had the greatest effect in quails during the finisher phase (Chapter 5). The results suggest that in general, larvae meal from both Dipteran species might decrease the occurrence of diseases caused by Gram-positive bacteria. Black soldier fly larvae meal had no effect on serum bactericidal activity against the Gram-negative bacteria, *E. coli*, in unchallenged quails (Chapter 4). Similarly, in broilers, BSF and CC larvae meal did not increase the serum bactericidal activity against *E. coli* (a bacterium they were not challenged with). Since an increase in serum bactericidal activity against *Salmonella* was detected in BSF and CC-fed chickens shortly after infection with *Salmonella* took place, it demonstrates that larvae meal enables the immune system to cascade a greater response against a Gram-negative pathogen shortly after infection with the pathogen occurred (Chapter 6).

Feeding BSF larvae meal to unchallenged quails did not affect selected bacterial counts in the ceca. However, when broilers were challenged with *Salmonella* Enteritidis, CC larvae meal were most effective in reducing *Salmonella* counts in the ceca of broilers that were found to be significantly lower than the control group at the time of slaughter. This phenomenon will help reduce the chances of carcass contamination during the slaughtering process. The *in vivo* antimicrobial activity of CC meal was slower than the activity of the antibiotic (oxytetracycline) used in the study. Oxytetracycline exhibited strong activity a few days after the infection occurred, but its activity decreased with time. Unfortunately, the BSF larvae meal did not significantly reduce *Salmonella* counts *in vivo*. *In vitro* results indicated that peptides extracted from CC larvae and larvae meal had strong bactericidal activity against *E. coli*, but a slightly lower activity against *Salmonella* Enteritidis were recorded. Results also showed that activity against *Salmonella* was greater for CC larvae meal compared to BSF larvae meal, partly explaining the *in vivo* results.

Neither of the larvae meal sources had any major effect on any of the haematological parameters, serum biochemical parameters or organ indices measured in any of the trials, indicating that larvae meal has no adverse impact on the physiological status of the animal. Black soldier fly larvae meal increased α2-globulin proteins in the serum of quails. Alpha-2-globulins are often associated with inflammation. However, certain α2-globulin proteins scavenge free radicals (Cray 2009), exhibit bacteriostatic properties (Eaton *et al.*, 1982),

inactivates toxins (Borth, 1992) and removes enzymes released during injury (Cray *et al.*, 2009). Therefore, since all quails in the study appeared healthy, increased α2-globulin levels in their serum can be an indication that BSF larvae meal holds immunostimulatory properties in this regard – this aspect warrants further research.

The inclusion of fish offal to the rearing substrate of BSF larvae increased the omega-3 (n-3) fatty acid levels in BSF larvae meal. The change in n6-n3 ratio had a moderate impact on the immunomodulatory properties of BSF larvae meal. Larvae meal from larvae reared on fish offal elicited a greater cellular immune response and higher lysozyme concentrations, whereas BSF larvae meal from larvae reared only on chicken feed provoked a greater secondary humoral immune response (Chapter 5). Therefore, it is possible that the substrate used to rear larvae on not only affects the nutrient composition of the larvae meal, but the immunomodulatory properties of the larvae meal can also be influenced or manipulated by the type of substrate used.

To conclude, BSF larvae meal and CC larvae meal can be used up to a 10% inclusion levels in diets of broilers and quails. In all three studies, larvae meal had no negative effects on any of the growth parameters, immune parameters, haematological or biochemical indexes measured. Larvae meal from both dipteran species either had immunostimulating effects on immune parameters measured, or no effect, but no negative effects were detected in the current study. Since the CC larvae are effective in breaking down animal offal to produce a high-quality protein source, very similar to fishmeal, with active *in-vivo* antimicrobial and immunostimulatory properties, it justifies further research on the safety risks arising from the consumption of eating livestock that was fed CC larvae meal. For example, the possible occurrence of prions, viruses, or bacterial pathogens in CC meal reared on infected offal should be explored. Suppose there is an indication of a carry-over effect of prions or other dangerous substances from the larvae meal down the food chain to humans. In that case, CC larvae meal could be explored for its use as a protein source in pet feed.

More research is warranted to determine what factors (substrate, age, body composition etc.) influence AMP expression in larvae and the antimicrobial activity of larvae meal. Even though research indicates that the antimicrobial activity of AMPs from larvae can be enhanced when the larvae are challenged with a microorganism through mass injection (Lee *et al.*, 2020), this method might not be feasible when mass rearing larvae for larvae meal production. Correlations between substrate microbiome and AMP production should be further explored, especially in CC larvae since research on AMPs in this species is scarce. A possible link between protein content in larvae ass AMP concentration should also be investigated. There is also a need for more trials with immunosuppressed poultry using dietary larvae meal. Results from immunosuppressed trials will create a better understanding of the real immunostimulatory value of larvae meal, especially when used in overcrowded production houses with poor biosecurity.

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